Bacterial and fungal infections: evolving towards molecular pathogen diagnostics

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Aan mijn ouders

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List of abbreviations

ACCP	American college of chest physicians
AST	antibiotic susceptibility testing
bp	base pair
BSI	bloodstream infection
CAP	community-acquired pneumonia
cfu	colony-forming unit
CLSI	clinical and laboratory standards institute
CoNS	coagulase-negative staphylococci
CSF	cerebrospinal fluid
Ct	cycle threshold
EPIC	European prevalence of infection in intensive care study
FISH	fluorescence in situ hybridization
FRET	fluorescence resonance energy transfer
GPC	Gram-positive cocci
ICU	intensive care unit
IFI	invasive fungal infection
LOD	limit of detection
MALDI-TOF MS	matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MIC	minimal inhibitory concentration
MLS	macrolide, lincosamide, and streptogramin antibiotics
MRSA	methicillin-resistant *Staphylococcus aureus*
NAT	nucleic acid-based technology
NPV	negative predictive value
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PPV	positive predictive value
ROC	receiver operating characteristic
SCCM	society of critical care medicine
SCOPE	surveillance and control of pathogens of epidemiological importance study
SIRS	systemic inflammatory response syndrome
SPS	sodium polyanethol sulfonate
UTI	urinary tract infection
WBC	white blood cell

Chapter 1

General introduction & outline of the thesis

1. Preface

Infectious diseases are caused by microorganisms such as bacteria, viruses, fungi or parasites. Some of these organisms constitute the “normal flora” or microbiota present and are participating in the metabolism of food products, the protection against pathogenic microorganisms, and the development and stimulation of the immune system. However, some of them can become pathogenic, for instance when introduced in normally sterile environments such as the blood, or in case of suppression of the immune system. Besides these opportunistic pathogens (e.g., *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Acinetobacter*spp.), strict pathogens exist that do not belong to the normal flora, and can cause disease (e.g., *Mycobacterium tuberculosis*, *Vibrio cholera, Neisseria gonorrhoeae*). Clinical symptoms associated with infectious diseases are mostly not disease-specific or not microorganism-specific and often include general signs such as fever, loss of appetite, fatigue and muscle aches. Therefore, laboratory tests of different body fluid samples (e.g., blood, urine, cerebrospinal fluid (CSF)) are used for the detection and determination of the causative agent. Traditional culture-based methods in the clinical microbiology laboratory are time-consuming, and are associated with a low sensitivity in case of slow-growing or fastidious microorganisms. In addition, the sample volume[1-5], the time from sampling to incubation[6-9], and the use of antibiotics or antifungal therapy can decrease the sensitivity of cultures[10-13]. On the other hand, the specificity of cultures can be hampered by the occurrence of false-positives due to contamination[14]. The rapid and accurate diagnosis of the etiologic agent in infectious diseases is essential for the adequate treatment of the patient. In addition, the simultaneous determination of the microorganisms’ antimicrobial susceptibility pattern is strongly needed for the guidance towards pathogen-tailored therapy.

The aim of this thesis was to contribute to a more rapid, sensitive and specific diagnosis of bacterial and fungal infections through the development of rapid real-time polymerase chain reaction (PCR)-based assays for the detection and identification of clinically important bacteria and fungi. In the present chapter, an overview will be given about the diagnostic tools, more specifically real-time PCR-based assays in the current clinical microbiology laboratory. Throughout the remainder of the thesis, experiments will be described in which we evaluated, developed and optimized methods for the isolation and purification of bacterial and fungal DNA from whole blood, blood cultures and urine specimens, the detection and identification of clinically relevant bacteria and fungi from blood cultures and urine specimens, and the genotypic determination of antibiotic resistance in staphylococcal blood culture isolates.

2. Bloodstream infections

Bloodstream infections (BSI), characterized by the invasion of microorganisms in the bloodstream, are a major cause of death all over the world. This life-threatening condition can be subdivided into bacterial and fungal BSI. In BSI, pathogens are disseminated throughout the body, and this is mostly caused by a primary focus of infection after trauma, or in intravascular devices, or in an organ system initially presenting for instance as a urinary tract infection or respiratory infection. In the United States, approximately 750,000 patients develop bacterial or fungal BSI annually, accompanied with a mortality rate ranging from 20 to 70%[15-18]. In Europe, it is estimated that each year approximately 135,000 patients die due to sepsis-associated complications[19]. A recent report by Engel *et al.* presented sepsis as the third most common cause of death in Germany[20]. Within The Netherlands, the annual admission of patients suffering from severe sepsis and septic shock is estimated to be 15,500 and 6,000, respectively[21]. The rapid diagnosis and management of BSI is critical to successful treatment. Inadequate antibiotic therapy is associated with higher mortality rates[22, 23], the appearance of antibiotic resistance in intensive-care units (ICUs)[24], and longer hospitalization lengths of stay[25].

2.1 Bacterial bloodstream infections

The presence of bacteria in the blood, defined as bacteremia, was first recognized by Libman in 1897. Two children, presenting with bloody diarrhoea were diagnosed of having streptococcal infection[26]. The occurrence of pathogenic bacteria in the bloodstream can cause severe damage to the body by eliciting a systemic inflammatory immune response, called Systemic Inflammatory Response Syndrome (SIRS). The term was introduced in 1992 at the American College of Chest Physicians/Society of Critical Care Medicine (ACCP/SCCM) Consensus Conference and provided a reference for the complex findings that result from a systemic activation of the innate immune response, regardless of cause[27]. SIRS is considered to be present when the following clinical symptoms are present: body temperature higher than 38°C or lower than 36°C, heart rate higher than 90 beats/min, hyperventilation evidenced by respiratory rate higher than 20 breaths/min or $PaCO_2$ lower than 32 mmHg or white blood cell (WBC) count higher than 12000 cells/mm^3 or lower than 4000/mm^3 [27]. The diagnosis of SIRS in combination with proven infection is called sepsis. Severe sepsis is defined as sepsis with organ dysfunction, hypoperfusion or hypotension. Septic shock is the final stage, in which severe sepsis is associated with hypotension despite adequate fluid resuscitation.
Risk factors for the development of BSI include age, underlying diseases, invasive procedures and immunosuppression[28]. Furthermore, the outcome of

bacteremia can be dependent on the source and the type of microorganism present in the blood. A nationwide surveillance study (Surveillance and Control of Pathogens of Epidemiologic Importance, SCOPE) in US hospitals found 65% of Gram-positive and 25% of Gram-negative bacteria as etiologic agent of nosocomial BSI[29, 30]. Coagulase-negative *Staphyloccus* spp. (CoNS), *S. aureus* and enterococci were the most common isolated pathogens[29]. Both, the recognition of clinical signs as well as the detection of the causative agent are extremely important in the acute phase of BSI. Hence, a timely and adequate treatment for patients with severe sepsis or septic shock will have a positive impact on outcome[25, 31, 32]. It was shown that the administration of inadequate empirical antimicrobial therapy in septic patients was associated with a higher mortality rate, and also with a longer hospitalization length of stay[23, 33]. In this perspective, current culture-based methods do not fulfil the wishes of the clinic since at least 24 to 72 hours are needed for the confirmation of an infectious etiology, the identification of the pathogen, and determination of its antimicrobial resistance profile[34]. Numerous attempts have been made for the improvement of diagnosis of BSI and continue to be developed and evaluated[34]. Though, until now there is not one alternative diagnostic tool capable of totally replacing the blood culture-based approach.

2.2 Fungal bloodstream infections

Invasive fungal infections have become a major contributor to ICU-associated infections. One outcome measure of the European Prevalence of Infection in Intensive Care (EPIC) study focused on the most frequently observed microorganism, and revealed the prevalence of fungi in 17% of ICU infections[35]. Similar reports on nosocomial infections in ICUs were performed in the US, and found fungi as causative agent in 9.5 up to 12% of the bloodstream isolates[29, 36]. The most important risk factors include immunosuppression, use of broad-spectrum antibiotics, central venous catheters and recent major surgery[37]. The vast majority of nosocomial fungal infections are caused by *Candida* spp., and are associated with a high mortality and morbidity[38, 39]. Data originated from the 2004 Surveillance and Control of Pathogens of Epidemiological Importance (SCOPE) study defined *Candida* spp. as the fourth most common cause of all hospital-acquired BSI in the US, and the third most common cause of hospital-acquired BSI in the ICU[40]. The most occurring causative species include *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei*[41]. The prevalence of these opportunistic pathogens, particularly in this group of critically ill patients, emphasizes the importance of an early and accurate diagnosis. Moreover, the empirical treatment is associated with an increased risk for drug toxicity and antifungal resistance. The diagnostic sensitivity of culture-based and histological techniques is often not sufficient

because some fungi are difficult to culture, and are rarely recovered from clinical specimens. This lack of sensitivity has been shown in patients with chronic disseminated candidiasis and invasive aspergillosis, in which less than 50% of the blood cultures were positive[42]. Besides, culturing is time-consuming and can take up to three weeks[43]. Many efforts have been made to defeat these shortcomings and alternative methods based on the detection of fungal antigens and pathogen-specific gene signatures are currently reported as most promising molecular diagnostics tools[44-48].

3. Blood cultures: the gold standard

Blood cultures are the "gold standard" of BSI diagnosis and are based on the detection of viable microorganisms in the blood. Whenever microbial growth has occurred, the positive blood culture is used for Gram staining, culture on agar plates, biochemical testing and antibiotic susceptibility testing (AST). The accurate identification of the causative pathogen and the determination of the microorganisms' antimicrobial profile are essential in the management of BSI, since both parameters are of significant importance for the guidance of antibiotic therapy. Therefore, both aspects can be seen as the strength of the current gold standard, because until now no other technique can offer both diagnostics parameters. Blood cultures have a central role in the clinical microbiology laboratory. In the past, numerous studies have been conducted to define the factors influencing the sensitivity of the procedure as well as to establish the most optimal conditions for blood culture processing. The current Clinical and Laboratory Standards (CLSI) guidelines provide the general principles and procedures for blood cultures from patients who are suspected of having bacteremia or fungemia[4]. The continuous optimization of various technical elements in blood culture processing such as automated detection of growth, or enhancement of culture media has lead to major improvements of the diagnostic performance. Though, several factors exist limiting the medical value of blood cultures.

Diagnostic yield

One of the most determining factors in the detection of pathogens is the blood volume[1, 2]. Studies of adult patients have reported numbers between 1 and 100 colony-forming units per millilitre (cfu/ml) during bacteremic episodes[49-51]. Pediatric patients are thought to have higher numbers of bacteria in the blood[49], however, reports also showed the occurrence of low-level bacteremia (≤10 cfu/ml) in 60-70% of their population[52]. The diagnostic yield is directly related to the volume of blood sampled, as presented in several studies of both adult[3, 53, 54] and pediatric patients[55, 56]. As recommended in the CLSI guidelines, 20 to 30 ml

of blood per culture set should be drawn in adults[4, 51]. This can be particularly problematic for pediatric patients, for whom mostly inadequate sample volumes can be obtained.

Non-cultivable pathogens and antimicrobial therapy

A second limitation of blood cultures is the lower sensitivity for slow-growing and fastidious microorganisms. These include pathogens involved in systemic diseases such as Whipple's disease (*Tropheryma whipplei*), bartonellosis (*Bartonella* spp.), Q fever (*Coxiella burnetii*) and rickettsiosis (*Rickettsia* spp.)[57, 58]. Many of these microorganisms can also be found in blood culture-negative infective endocarditis[59]. Furthermore, pathogens causing community-acquired pneumonia (*Legionella pneumophila*, *Chlamydia pneumonia*, and *Mycoplasma pneumonia*) can render negative blood cultures[60]. The occurrence of these fastidious and slow-growing pathogens will cause a serious delay in the identification process, which can lead to prolonged and inadequate empirical antimicrobial therapy. Another potential interfering factor resulting in negative blood cultures is previous antimicrobial therapy, which can give rise to false-negative results[10, 11, 13]. This can be particularly the case in patients receiving prophylactic antibiotics. In these cases, the inhibitory effect of antibiotics must be prohibited by using the most optimal culture conditions.

Turnaround time

A third reason limiting the clinical value of blood culture is the delay in time to results. After the detection of microbial growth in a blood culture bottle, Gram staining is performed and the positive blood culture is used for further culturing on plate. The median time to positivity is approximately 15 hours (range 2.6-127 hours)[61-63], and subsequent subculturing takes an additional time of at least 24 hours. Of course, the bacterial or fungal load, the type and characteristics of the pathogen are factors influencing these time ranges. Rapid Gram staining and biochemical tests can offer an initial insight about the etiologic agent within one hour after growth detection. However, more time is needed for the final identification and AST of the causative agent.

4. Molecular pathogen diagnostics

Molecular techniques have become a growing field of interest for the detection and identification of bacteria and fungi present in bloodstream infections. This is mainly due to the limitations associated with the conventional culture-based approach which include the delay between sampling and analysis, the need for bacteriological expertise, the personnel workload and the insensitivity for

fastidious or slow-growing microorganisms. Major evolutions have been made so far, but even today, the ideal technique, in which simultaneous pathogen identification and determination of the antimicrobial susceptibility pattern is provided, does not exist. Throughout the years, many technologies were evaluated and an overview of the different approaches is given in Figure 1. The diagnosis of bacterial or fungal BSI can be based on the detection of pathogens from cultured specimens (blood culture) or directly from whole blood, plasma or serum. The latter category demands a higher performance capacity in terms of detection limit because of the potential low number of microorganisms in the blood. The identification of the etiologic agent without the need for prior culturing would reduce the turnaround time drastically, and would enable quantification of the bacterial or fungal load. The next section will highlight existing and more recent technologies for the detection and identification of pathogens that are based on the detection of nucleic acids (real-time PCR, fluorescence in situ hybridization (FISH)) or proteins (matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)). These molecular assays may offer several advantages such as rapidity (i.e. within a few hours), require small sample volumes, enable high-throughput, and reduce microbiological workload.

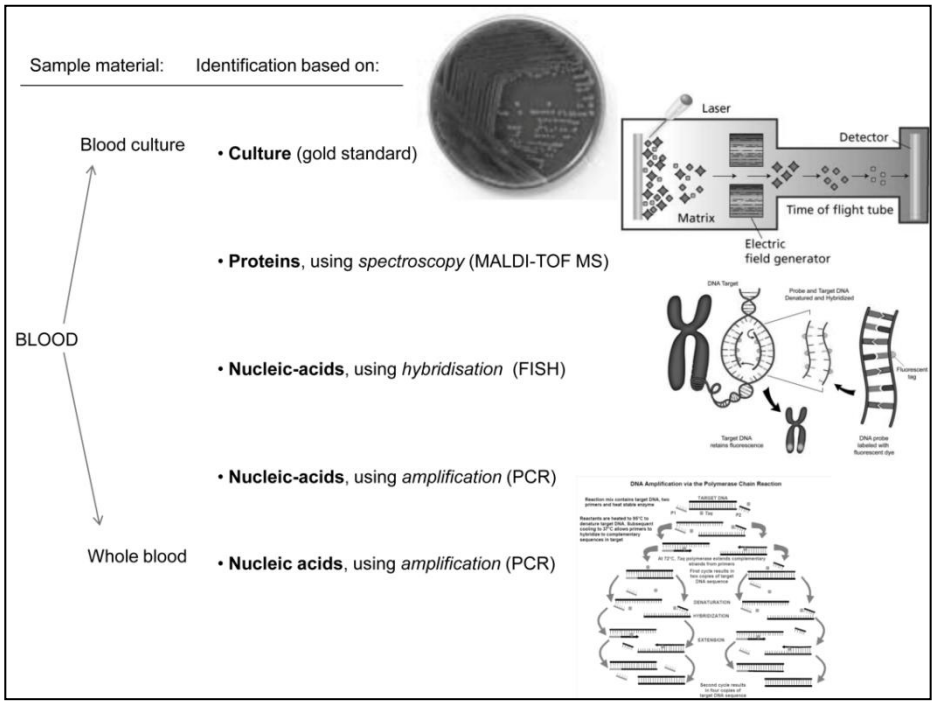

Figure 1. Overview of the most frequently used techniques for the detection and identification of bacterial and fungal pathogens involved in bloodstream infections. This figure was redrawn from Peters, 2004[64].

Protein-based identification techniques

Protein-based identification uses vibrational spectroscopy for the determination of the protein composition of a sample, and a widely discussed application is matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). In clinical microbiology, MALDI-TOF MS is used to analyze specific peptides or proteins which are directly desorbed from microorganisms[65]. The taxonomic classification of individual microorganisms is based on the existence of unique proteomic fingerprints that allow species-specific identification, and was first reported about 30 years ago[66]. Previous studies showed that MALDI-TOF MS can be used for the identification of Gram-positive bacteria[67-72], Enterobacteriaceae[73], non-fermenting bacteria[74-77], mycobacteria[78-80], anaerobes[71, 81], and yeasts[82, 83].
MALDI-TOF MS allows a very rapid, i.e. within minutes, identification of the microorganism, but currently involves a prior culturing step to increase the number of microbial cells for analysis. Recent studies investigated the potential use of positive blood cultures as starting material for MALDI-TOF MS[84-89]. Whereas bacterial identification from colonies can be performed without any sample preparation, pre-treatments are suggested in case of fungal identification. Using blood cultures as specimen obligates the need of eliminating human red blood cells in order to prevent interference during MS analysis. As suggested by Drancourt *et al.* the most optimal protocol for processing of blood cultures remains to be developed and evaluated to enable standardisation and automation[90]. The main problems encountered with MALDI-TOF MS analysis are the difficulty in identifying mixed organisms[86, 87, 89] and viridans *Streptococcus* spp. organisms[86, 87]. Although suggested as a candidate method replacing traditional techniques for microbial identification[91], one has to keep in mind that successful MALDI-TOF MS analysis is dependent on the microbial load (10^7-10^8 cfu/ml) requiring pre-culturing of the blood. Apart from the identification process, the role of MALDI-TOF MS in the determination of antibiotic resistance needs further investigation. First reports presented the successful identification and discrimination of methicillin-resistant *S. aureus* (MRSA)[92, 93]. However, the search for other antibiotic resistance determinants remains to be elucidated.

Nucleic acid-based identification techniques

Nucleic acid-based technologies (NATs) have been introduced for the rapid identification of microorganisms and are based on the detection of microorganism-specific DNA or RNA sequences. Widely studied NATs that have been used for the diagnosis of infectious diseases include detection assays based on hybridization or amplification of target sequences. An example

of a hybridization-based assay is fluorescent in situ hybridization (FISH), in which fluorochrome-labelled oligonucleotide probes targeted to rRNA are visualised using microscopy. In previous studies, FISH assays were developed for the detection of *Chlamydia* spp.[94], *Enterococcus* spp.[95], *Pseudomonas aeruginosa*[96], *Helicobacter* spp.[97], *Streptococcus* spp.[98], *Staphylococcus* spp., *Escherichia coli*[96], *Candida* spp., and other Enterobacteriaceae[95, 99]. FISH allows the identification of bacteria and yeast from blood cultures within 2 to 5 hours. The usefulness of the procedure is largely dependent on the type of microorganism since the permeabilization of the cell wall during sample preparation is a critical step in the process. Next, the technology is highly specific, but depending on probe design and hybridization conditions. Because of the currently limited probe repertoire, species-specific identification is not possible for all bacterial species. In a study of Peters *et al.* identification at genus or family level and at species level was shown in 91% and 79%, respectively[100]. These results confirmed earlier data obtained by Kempf *et al.* in which the usefulness of FISH as a diagnostic test was recommended[99]. Though, the panel of probes included should be well considered and designed in relation to the pathogens present in a specific patient setting. Among the amplification-based NAT, the polymerase chain reaction (PCR) and its derivatives are one of the preferred methods. This branch of molecular techniques will be discussed in the next paragraphs. First, the importance of sample preparation will be discussed, followed by the opportunities associated with real-time PCR assays.

5. Nucleic acid extraction

In order to release and concentrate microbial DNA, the extraction and purification of bacterial and/or fungal nucleic acids from patient specimens involves a three-step procedure including the lysis of the microorganism, followed by the selective binding and elution of the bacterial and/or fungal DNA. Independent of the downstream NAT application, the purification of the bacterial or fungal nucleic acids from the sample material is a critical step in the whole process[101]. The low concentration of pathogenic DNA in relation to the high amount of human DNA[102], the presence of PCR-inhibitory compounds in patient specimens and/or culture media[103, 104], and the possible presence of contaminating bacterial or fungal DNA in reagents[105-108] are important challenges that are faced during the diagnosis of bacterial and fungal pathogens. Classical phenol/chloroform extraction followed by ethanol precipitation is toxic, and impractical for the processing of large numbers of samples. During recent years, many commercial kits and automated DNA isolation instruments were developed and evaluated, and mainly use spin-

column-based extraction (e.g., QIAamp DNA Mini Kit (Qiagen, Hilden, Germany)).
The popularity of the latter is mostly based on the low costs and the easiness to use, only requiring standard equipment which is available in most routine laboratories. The principle of DNA extraction using column purification is i) release of the nucleic acids through denaturation of the proteins with chaotropic salts, ii) separation of the nucleic acids through adsorption onto a silica gel membrane, iii) washing with high-salt-concentration buffers to remove denatured proteins and other compounds, iv) elution of the bound DNA with a low-salt-concentration buffer. In a study by Rantakokko - Jalava and Jalava, the results indicated that no single DNA extraction method is optimal for the detection of all microorganisms, and for application to every patient specimen[109]. Indeed, for the extraction of fungal nucleic acids, it became clear that specific protocols need to be used because of the difficulty in breaking the cell wall[43, 110]. Therefore, as more insights were gained concerning the different types of patient specimens and the subsequent variable presence of inhibitory compounds, methods were fine-tuned according to the application.

Detection in direct patient specimens (without enrichment)

In order to facilitate a rapid detection of the causative infectious agent, processing of patient specimens without prior bacterial/fungal enrichment, i.e. direct specimens are preferred. Nucleic acid extraction from direct patient specimens such as whole blood, plasma or serum can be difficult because of the high amount of human DNA versus the low amount of bacterial DNA. In addition, whole blood contains many PCR inhibiting substances which emphasize the need for further processing[111]. Commercially available DNA extraction kits were evaluated and compared in terms of performance between with or without proteinase K digestion[112], manual or automated nucleic acid extraction[113], binding-plate or filter-plate format or magnetic bead format[114]. More recently, commercial whole blood assays such as MolYsis (Molzym, Bremen, Germany), Looxster (SIRS-Lab, Jena, Germany) and Septifast (Roche Diagnostics, Mannheim, Germany) DNA isolation Kit were developed and included a pre-treatment protocol together with a DNA extraction protocol. The focus of these kits is the removal of interfering host DNA and the enrichment of pathogen DNA. In a study performed by Handschur *et al.*, results showed that the removal of human DNA eliminated unspecific signals occurring in the 16S rDNA real-time PCR[115]. Wiesinger - Mayr *et al.* compared different commercially available assays (Looxster, MolYsis Kit, SeptiFast, standard EasyMAG (BioMérieux, Marcy l'Etoile, France) DNA isolation) with a semi-automated EasyMAG protocol, supplemented with pre-processing steps involved in human DNA elimination[116]. Together with the Looxster kit, the

modified EasyMAG protocol generated the most sensitive results, achieving a detection limit of 10^1 to 10^2 bacterial cells per ml of whole blood. Besides whole blood, reports also showed the applicability of these tests to oral samples such as saliva[117, 118], and other clinical samples such as tissue biopsy, synovial fluid, pleural fluid, and CSF specimens[109]. The addition of pre-processing steps (i.e. human DNA elimination with MolYsis or Looxster) removed at least 90% of the human DNA present in oral samples, whereas bacterial DNA recovery ranged between 35 and 50%[117, 118]. The data presented by Rantakokko - Jalava and Jalava indicated that no single method is optimal for the detection of all bacteria, or for all patient specimens[109].

Detection in cultured patient specimens

Cultured patient specimens such as blood cultures consist of a high bacterial or fungal load, but inhibitory substances such as the anticoagulant and anticomplementary agent sodium polyanethol sulfonate (SPS) can influence downstream applications like PCR[119]. Millar *et al.* compared a series of commercially available (Qiagen QIAmp Blood kit (Qiagen, Hilden, Germany), Roche high PCR template preparation kit (Roche Diagnostics, Mannheim, Germany), Puregene DNA extraction kit (Qiagen, Hilden, Germany)) and in-house assays (boiling, glass beads/sonication and wash/alkali/heat lysis) for blood cultures, and presented a wash/alkali/heat lysis method as the most sensitive, reproducible and cost-effective DNA extraction method[120]. Besides the high yield of bacteria and/or fungi present in positive blood cultures, a more rapid identification and antibiotic susceptibility pattern of life-threatening pathogens such as MRSA[121-123]*and Streptococcus pneumonia*[124, 125] can be achieved. More recently, a study was performed in which six DNA extraction protocols were compared to accelerate the detection of *S. aureus* and coagulase-negative staphylococci (CoNS) from two different types of blood culture materials, i.e. BACTEC (Becton, Dickinson and Company, Sparks, US) and BacT/ALERT (BioMérieux, Marcy l'Etoile, France)[126]. The most sensitive technique achieved a detection limit of 10 cfu/ml in BacT/ALERT material, whereas 100 cfu/ml could be detected in BACTEC blood culture material. In addition, they tested the effect of reduced blood culture incubation times in combination with the most sensitive extraction method, and showed that an initial *S. aureus* or CoNS load of 1 cfu/ml could be detected after five hours of incubation, compared to 28 hours with conventional methods. These results were also shown in a paper presented by Gebert *et al.*[127] and revealed a significant reduction in time to results. This is also of importance for another group of microorganisms, i.e. fungi, which could also benefit from accelerated detection, since full identification can take more than 72 hours[128].

6. Nucleic acid amplification

The PCR constitutes one the most important nucleic acid amplification techniques and was developed by Mullis and Faloona in 1987[129]. PCR is used for the in vitro-amplification of DNA, which functions as a molecular fingerprint[130]. The DNA is copied by a heat-stable polymerase in the presence of nucleotides, buffers and primers (Figure 2).

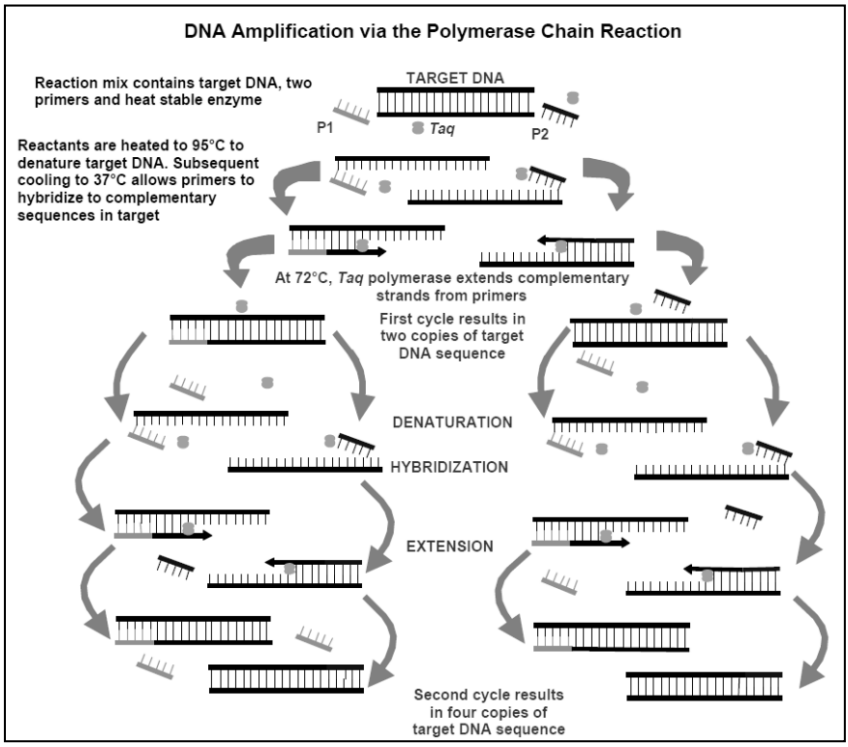

Figure 2. The basic mechanism of the polymerase chain reaction (PCR). This figure was redrawn from ORNL-DWG91M-17476.

Primers are short DNA oligomers that are complementary to the ends of a target sequence. The primers hybridize to their complementary regions of DNA, and subsequently DNA polymerases extend the DNA strands, producing a copy of the DNA. Each copy of DNA serves as another template, resulting in an exponential amplification of the original DNA sequence. In the clinical microbiology laboratory, PCR was introduced as a promising technique for the molecular diagnostics of infectious diseases[131-133]. Both unique and eubacterial (e.g., 16S rDNA) DNA sequences of microorganisms can be used for their identification, or can be helpful in the discrimination between certain types of microorganisms. The development of real-time PCR, which combined

conventional PCR with fluorescent probe detection of the amplified DNA product, offered major opportunities in the field of molecular pathogen diagnostics[134-138]. In real-time PCR, nucleic acid amplification and detection are performed within the same closed vessel, minimizing the risk for contamination. In addition, compared to conventional PCR, the ease of performance, speed and high sensitivity and specificity levels have made real-time PCR an appealing alternative approach for the conventional culture-based diagnosis of infectious diseases.

The most traditional method for the visualization of double-stranded DNA product is SYBR Green nucleic acid detection. SYBR Green assays are not specific, but can provide melting curve analysis for the determination of the melting temperature of the different amplification products. Instead, the use of fluorescent probes, capable of fluorescence resonance energy transfer (FRET)[139], allowed sensitive and specific detection of target DNA. The first fluorescent probes were TaqMan probes, or also called 5' nuclease probes, which are short oligonucleotides containing a 5' fluorescent dye and 3' quenching dye. The fluorescent and quenching dye have to be separated by the Taq polymerase in order to generate a light signal. This can only be achieved when the probe binds to a complementary strand of DNA, which underlines the specificity of this technology. The accumulation of amplification product can be monitored in real time and generates a change in signal, which can be related to the amount of DNA present during each cycle[135]. Other types of nucleic acid detection using fluorescent probes are molecular beacons and FRET hybridization probes[140].

6.1 Identification of bacteria and fungi

The high sensitivity and specificity together with a short turnaround time for results, and the ease of performance make real-time PCR a promising replacement method for conventional culture-based identification methods[140]. Numerous studies describe real-time PCR-based assays for the identification of bacteria and fungi, and they can be based on broad-range, pathogen-specific or multiplex detection. Pathogen-specific assays are targeted to a single bacterium of fungus, and have a more limited role in diagnostic settings because of the high variety of pathogenic microorganisms responsible for infectious diseases[34]. However, in some diseases such as typhoid fever, the rapid identification of *Salmonella typhi* was established using species-specific real-time PCR detection[141, 142]. Also, in previous studies pathogen-specific assays were developed for the detection of *Staphylococcus epidermidis*[143], *S. aureus*[144, 145], MRSA[121-123, 146-149], *S. pneumonia*[150-153], *Neisseria meningitides*[154, 155], *M. tuberculosis*[156-158], *Brucella* spp., *C. albicans*[159], and *Aspergillus* spp.[160]. The main disadvantage is that they only can be used when a certain pathogen is

suspected, and laboratory confirmation is needed. In the diagnostic workup of patients with fever and suspected bloodstream infection broad-range detection or multiplex assays are preferred[128]. Universal targets such as the 16S rDNA gene or the 16S-23S rDNA gene interspacer region for bacteria[161-163], or the 18S and 28S rDNA gene for fungi[164, 165] are being used extensively for identification[166]. For instance, the 16S rDNA gene sequence is about 1550 base pairs (bp) long, composed of both variable and conserved regions, demanding the need for post-amplification sequencing[167]. Sequencing facilitates the identification of the etiologic agent, but no differentiation at the species or strain level is possible with this technique. Broad-range PCR in combination with sequencing has been applied to identify bacteria from blood[168-178], but this is laborious, rather expensive and time-consuming[128]. A review of Clarridge focused on the impact of 16S rDNA gene sequence analysis in clinical microbiology. They described both the opportunities and limits associated with this technology[167]. More recently, in a review of Sontakke *et al.* the past and current applications of broad-range 16S rDNA PCR for the diagnosis of bacterial infections are summarized and discussed[179]. A compromise between pathogen-specific and broad-range detection could be achieved with the development of multiplex PCR assays. Multiplex real-time PCR detection assays use one or more primer sets, eventually in combination with one or more fluorogenic oligoprobes for the amplification of multiple targets within a single reaction. Different strategies can be used, and one of them comprises the use of different primer sets that are specific for one microorganism[180-182]. On the other hand, one broad-range primer set can be used in combination with different fluorescently labelled probes complementary for a panel of bacterial and/or fungal targets. Some studies reported the use of molecular probes for the classification of Gram-type[184], eventually in combination with pathogen identification[127, 183, 184], and achieved detection within three to six hours, while sensitivity and specificity ranged between 95% and 100% compared with the conventional culture-based method. Nowadays, many commercial systems offering a panel of clinically relevant bacteria and/or fungi have been developed and evaluated. One of the most described commercial assays available for the identification of bacteria and fungi is the Septi*Fast* (Roche Diagnostics, Mannheim, Germany) test. The kit is intended for the identification of up to 25 microorganisms from whole blood using real-time PCR coupled to melting curve analysis[185]. The latest studies, both in adults[186-189] and children[190] demonstrated the potential of Septi*Fast* to be of added value to blood cultures, by reducing the time to results and adding diagnostic yield, especially in antibiotic pre-treated patients. Other similar testing platforms are Prove-It Sepsis (Mobidiag, Helsinki, Finland), which combines broad-range PCR with microarray hybridization for the detection of 60 bacteria and 13 fungi from blood cultures. Gaibani *et al.* found an agreement of more than 90% (when the microorganisms

found in blood culture were included in the Prove-It Sepsis panel) between Prove-It Sepsis and blood cultures[191]. The SepsiTest (Molzym GmbH, Bremen, Germany) consists of a broad-range real-time PCR (absence of presence of microorganisms) combined with sequence identification, capable of detecting more than 345 bacteria and fungi from whole blood specimens. In a study performed by Wellinghausen *et al.* results showed that the concordance of PCR and blood culture for positive and negative samples was 86%[192]. The VYOO Kit (SIRS-Lab GmbH, Jena, Germany) uses a multiplex PCR followed by microarray analysis, enabling the detection of 34 bacteria and seven fungi from whole blood samples. Yet, no published data on clinical experience exist, only two abstracts were reported on 24 (Sachse, unpublished data) and 63 (Bloos, unpublished data) patients[193]. Application of VYOO did only result in 15% of modifications of antimicrobial therapy and positive PCR was not associated with increased mortality[193]. Overall, the turnaround times of the described commercial assays ranged between 3.5 hours to 7 hours, except for the SepsiTest, which involves sequence identification. Although the presented data showed the potential of newly developed molecular assays based on real-time PCR in terms of rapid pathogen identification, interventions studies are needed that focus on clinical experience, i.e. patient outcome and modification of antimicrobial or antifungal therapy.

Determination of the bacterial load

In clinical microbiology, real-time PCR assays are mainly used for the qualitative (i.e. absence or presence) detection and identification of microorganisms. However, the quantification of bacterial and/or fungal pathogens from direct specimens such as whole blood could enable correlation with important clinical parameters such as disease severity, therapy response, and outcome. Few studies have been performed to prove correlation between bacterial or fungal DNA load and disease severity. In a study focusing on patients with meningitis, meningococcal bacterial DNA load was found to be significantly higher in patients with severe compared to milder disease[194]. Also, bacterial DNA load was shown to be a diagnostic marker of pneumococcal infection in patients with community-acquired pneumonia (CAP)[195], and this was in concordance with another report by Kee *et al.*[196]. In certain diseases (e.g., urinary tract infections (UTIs), endocarditis) quantification of pathogens is essential because the diagnosis is based on clinical symptoms together with the bacterial load of the etiologic agent. For instance, the diagnosis of UTIs is based on semi-quantitative urine culture (reference standard) since different bacterial loads ($>10^3$ or 10^5 cfu/ml) are used to identify UTIs in different patient populations[197]. Also, discrimination may be possible between the presence of contaminants and causative pathogens. This can be relevant in samples in

which distinction is needed between normal flora and disease-causing microorganisms, such as in patients with corneal ulcer, as studied in a paper of Itahashi *et al*[198]. Quantification of pathogens has also been investigated in other patient specimens such as blood[195, 199] and CSF[200, 201]. One of the drawbacks described in these reports is the lack in distinction between viable and dead microbial cells. Following, the origin of the bacterial DNA is unclear, and may cause difficulties in interpretation when patients receiving antibiotics are monitored in time. After the administration of antibiotics, the amount of living bacteria is reduced, while bacterial DNA may persist[194, 202, 203]. Therefore, the clinical value of bacterial load determination (i.e. bacterial DNA) remains to be elucidated in terms of correlation with disease severity, patient outcome, and therapy response.

6.2 Detection of antibiotic resistance genes

Besides detection and identification of microorganisms, antimicrobial susceptibility testing (AST) of bacterial pathogens is one of the main functions of the clinical microbiology laboratory, and is essential for the guidance of antimicrobial therapy. Reports have shown that the administration of the appropriate antibiotics is correlated with a decrease in mortality[25, 32, 33, 204]. Conventional AST methods including agar dilution, broth microdilution, E-test and disk diffusion, still involve pure subculturing and therefore take up to 24 hours before initial results are known. To increase the rapidity and accuracy of susceptibility testing, genotypic methods have been introduced for the detection of antibiotic resistance genes[205-207].

Staphylococci

Throughout the years, many studies have focused on the genotypic detection of antibiotic resistance using in real-time amplification of known resistance genes, of which *mecA*, the gene for methicillin (oxacillin) resistance in staphylococci is most established in clinical practice[121, 208-214]. Methicillin resistance in staphylococci is conferred by the chromosomally located *mecA* gene, which encodes for an altered penicillin-binding protein PBP2a[215-217]. Production of PBP2a results in a lowered affinity for beta-lactam antibiotics. Both *S. aureus* and CoNS are known to accumulate more than one antibiotic resistance determinant, resulting in multi-drug resistant strains. Therefore, other antibiotics including penicillins, aminoglycosides, glycopeptides and macrolide, lincosamide, and streptogramin (MLS) antibiotics are also commonly used to treat infections caused by staphylococci, and are of particular interest for further implementation in a rapid real-time PCR assay. Resistance to penicillins is caused by the presence of the *bla*$_Z$ gene, which encodes for beta-lactamase, an

enzyme responsible for the hydrolysis of the beta-lactam nucleus[218]. Vancomycin, belonging to the group of the glycopeptides, is used to treat infections caused by multi-resistant Gram-positive microorganisms such as oxacillin-resistant staphylococci. *VanA*-type resistance is the most commonly encountered form and results in the production of an alternative D-ala-D-ala ligase, a cell wall precursor with a lowered affinity to glycopeptides[219, 220]. Resistance to aminoglycosides occurs mainly because of the presence of aminoglycoside-modifying enzymes that interfere with protein synthesis. The most encountered one is 6'-N-acetyltransferase-2''-O-phosphotransferase (AAC(6')-APH(2''), and is encoded by the *aac(6')-aph(2")* gene[220]. The presence of this gene results in phenotypic resistance to gentamicin, kanamycin, tobramycin, neomycin and amikacin[221]. Within the group of the MLS antibiotics different mechanisms of acquired resistance have been found in Gram-positive bacteria[220]. Target modification by methylases, which are encoded by erythromycin ribosome methylation (*erm*) genes, is the most common mechanism found. Another relevant possibility is the presence of efflux proteins, encoded by for instance the *msrA* gene. Presence of the *msrA* gene confers resistance to both macrolides and streptogramin B antibiotics (MS phenotype)[220, 222].

DNA microarray-based detection of antibiotic resistance genes can be used as a tool for the determination of a detailed antimicrobial resistance profile, as was presented by Perreten *et al.*[223]. They reported a DNA microarray capable of detecting 90 antibiotic resistance genes occurring in Gram-positive bacteria. A similar report by Frye *et al.* also presented a DNA microarray including 94 antimicrobial resistance genes in both Gram-positive and Gram-negative bacteria[224]. Although the presented microarrays have been shown to be efficient prototypes for the rapid screening of antibiotic resistance genes, the high costs associated with this technique limit the clinical applicability. From this perspective, small-scale microarrays have been developed, offering the detection of a panel of clinically relevant staphylococcal antibiotic resistance genes[225, 226]. Zhu *et al.* found correlations were more than 90% for detection of the phenotypic resistance in 415 staphylococcal isolates, while Strommenger *et al.* only tested 13 clinical isolates as a proof of concept, resulting in 100% concordance with phenotypic AST. Besides DNA microarrays, multiplex real-time PCR assays have been designed offering a similar panel of antibiotic resistance genes (e.g., *mecA*/methicillin resistance, *aacA-aphD*/aminoglycoside resistance, *ermA-B-C-msrA*/MLS resistance, *vatA-B-C*/streptogramin A resistance, *bla$_Z$*/penicillin, *vanA*/vancomycin)[227-231]. Overall, high correlations were found between genotypic and phenotypic analysis, though it has to be taken into account that novel or unknown resistance mechanisms may exist, harbouring the analytical sensitivity of the assay. For this reason, current genotypic antibiotic resistance detection assays have to be seen as an additive

to traditional AST testing, offering a faster detection of certain antibiotic resistances (e.g. methicillin resistance, vancomycin resistance) that are associated with a high clinical relevance.

7. Hypothesis and Outline of the Thesis

More than ever, molecular diagnostics is used as platform for the detection and identification of pathogens in the field of infectious diseases. Techniques included in this niche of diagnostic testing are ought to be rapid, sensitive, specific and robust. However, the successful application of molecular techniques is dependent on many factors. A wide variety of sample materials can be processed, although different sample preparations might be required. Analysis of whole blood would be the most favourable approach because of the relevant applicability in for instance urgent interventions at intensive care units (ICUs). Though, the complex mixture of human and bacterial or fungal DNA and other interfering compounds makes it the most challenging sample for designing a suitable handling protocol. Detection and identification of bacterial pathogens can be established using a wide variety of molecular techniques. An assay must be founded on a simple and straightforward technique, in combination with rapid en reproducible results. The main objective of this thesis was to contribute to a more rapid diagnosis of bacterial and fungal infections in whole blood and blood culture samples. To realize this goal, the whole process from sample collection to data interpretation, was carefully unravelled.

First, we evaluated a series of preanalysis sample treatment tools and DNA isolation protocols for whole blood samples. Pre-analytic kits have been developed for the removal of human DNA and the selective enrichment of bacterial DNA. Therefore, we determined the detection limits of four selected DNA extraction kits in combination with two novel pre-analytical sample treatment protocols for the isolation of methicillin-resistant *Staphylococcus aureus* (MRSA) DNA from whole blood **(Chapter 2a)**.

One of the limiting factors of whole blood can be the presence of low counts of circulating bacteria in the bloodstream. This is also influenced by patient- and infection-related characteristics. The level of implementation of molecular techniques will be dependent on these individual-specific features and will determine the potential of working with whole blood. Hence, we focused on the bacterial load in neonates in order to obtain better insights in the requirements needed for successful application of nucleic acid amplification techniques **(Chapter 2b)**.

Next, we aimed to design a rapid and clinically relevant molecular assay capable of the detection and identification of the most frequently encountered microorganisms causing bloodstream infections. Moreover, priority was given to those pathogens that could direct the choice of antibiotic therapy. The 16S

rDNA gene signature, present in all bacteria, was used for the design of fluorescently labelled probes. Using a real-time PCR-based platform supplemented with four species- and four genus-specific probes, we were able to identify important bacterial pathogens from positive blood cultures within two hours **(Chapter 3a)**.

Earlier data pointed out that besides bacteria certain fungi comprise a significant population found in blood cultures. Invasive fungal infections are particularly considered as cause of opportunistic infections in immunosuppressed patients. So, a rapid diagnosis and therapy is directly connected with patients' outcome. Gram-stained specimens presenting with yeasts were subjected to a real-time PCR assay containing probes for *Candida* spp., *Candida albicans*, *Candida glabrata*, *Candida krusei* and *Candida parapsilosis* **(Chapter 3b)**.

Both newly developed assays proved to be of relevant value in the diagnosis of bloodstream infections (BSIs). In an attempt to broaden the application field of these methods, we wanted to expand the bacterial pathogen identification assay with a semi-quantitative tool for diagnosis of infections requiring enumeration of the bacteria. Therefore, a semi-quantitative real-time PCR-based breakpoint was established to aid in the diagnosis of urinary tract infections (UTIs) **(Chapter 4)**.

Knowledge about the kind of causative pathogen is coherent to the determination of the antimicrobial resistance profile in order to direct the most suitable antibiotic therapy. In parallel to the identification of staphylococci, the most relevant antibiotic resistance phenotypes were genotypically determined in a real-time PCR assay. The development of such an assay, capable of identification and antibiotic susceptibility testing of the bacterial pathogen, would contribute to a more rapid diagnosis and therapy of BSI **(Chapter 5)**.

In the last chapter **(Chapter 6)**, a summary is made of the preceding findings and future perspectives are given.

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Chapter 2a

Evaluation of new preanalysis sample treatment tools and DNA isolation protocols to improve bacterial pathogen detection in whole blood

Wendy L. J. Hansen, Cathrien A. Bruggeman, Petra F. G. Wolffs
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Abstract

Two novel preanalysis sample treatment tools were evaluated in combination with four DNA extraction kits for the selective isolation of bacterial DNA from whole blood. The combination of performing a preanalysis sample treatment and using a larger sample volume increased the detection limit to 50 colony-forming units (cfu) per ml.

1. Introduction

New approaches, using molecular technologies are continuously being developed to improve the diagnosis of bloodstream infections. A critical issue in the success of the application of molecular methods is the sample treatment and/or nucleic acid isolation[1]. The low concentration of pathogens and the presence of PCR-inhibitory compounds in blood are important challenges that should be dealt with during sample treatment[2-6]. This could be done with so called preanalysis sample treatment tools, which combine selective enrichment of bacterial DNA from blood with integrated, highly efficient removal of PCR inhibitors. The aim of this study was to determine whether the addition of a preanalysis sample treatment to a selective DNA extraction protocol could improve the amplification and detection of bacterial DNA from whole blood samples.

2. Material and methods

MolYsis Basic (MolZym, Bremen, Germany) and Looxster (SIRS-lab, Jena, Germany) were evaluated as novel preanalysis sample treatment tools in combination with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), MagNA Pure LC Microbiology Kit M^{Grade} (Roche Diagnostics, Mannheim, Germany), MolYsis Complete Kit (MolZym, Bremen, Germany) and MagSi-DNA Isolation Kit for Blood (MagnaMedics Diagnostics, Maastricht, The Netherlands). A schematic overview of the performed analytical processes for whole blood spiked with methicillin-resistant *Staphylococcus aureus* (MRSA) is given in Figure 1. Bacterial DNA extraction was followed by amplification in a multiplex real-time PCR assay targeting three MRSA genes (*mecA*, *femA* and *sa442*)[7]. Real-time PCR was set up in a final volume of 50 µl with 2x Absolute^{TM} QPCR ROX (500 nM) Mix (Abgene, Epsom, United Kingdom). Primers and probes were purchased from Sigma-Genosys (Haverhill, United Kingdom) and Applied Biosystems (Nieuwerkerk a/d Ijssel, The Netherlands), respectively. Final reactions contained 0.6 µM of *mecA* primer and 0.3 µM of *femA* and *sa442* primer, 100 nM, 125 nM and 150 nM of *femA*, *mecA* and *sa442* probe respectively and 18.85 µl of template DNA. Optimal thermal cycling conditions were as follows: initial denaturation at 95°C for 15 min, 42 cycles of denaturation for 15 s at 95°C and annealing at 60°C for 1 min.

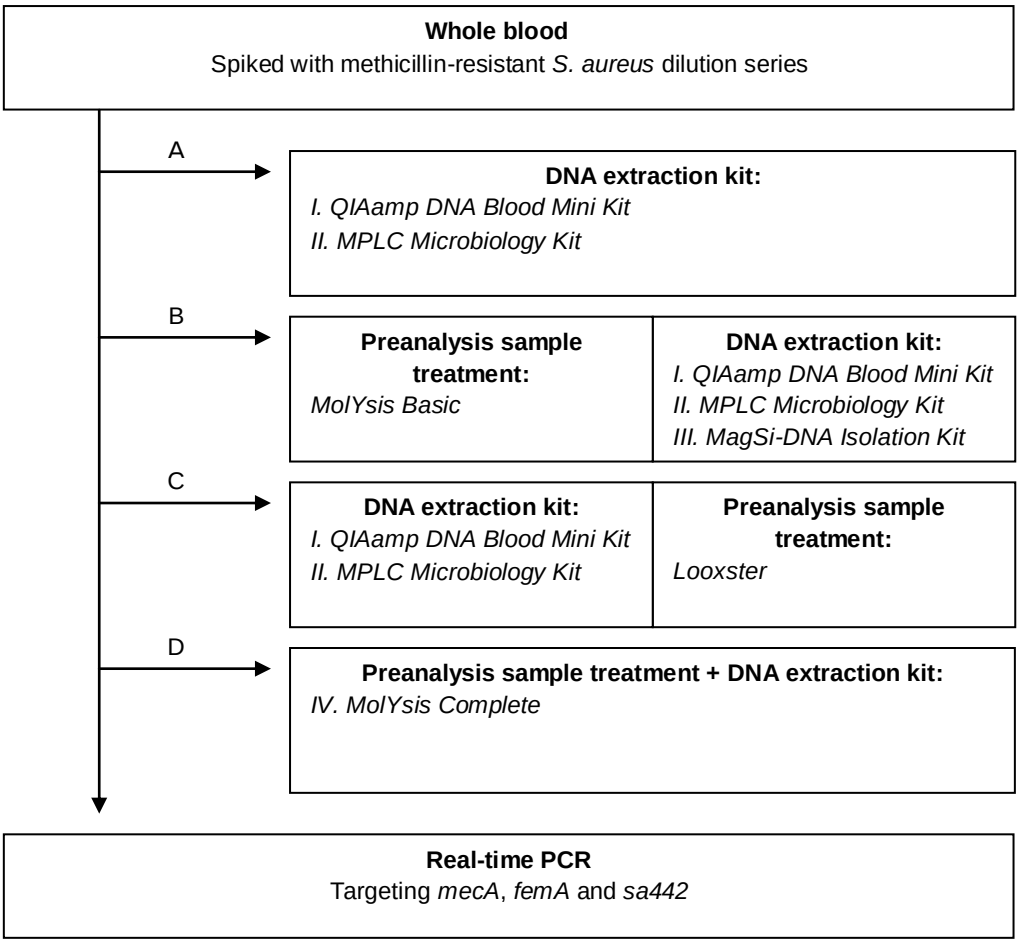

Figure 1. Schematic overview of the analytical process for whole blood samples. A, DNA extraction with two conventional kits, without preanalysis sample treatment. B, DNA extraction with two conventional kits and one novel kit, following preanalysis sample treatment with MolYsis Basic. C, DNA extraction with two conventional kits, followed by a preanalysis sample treatment with Looxster. D, DNA extraction with a novel kit, which combines all the steps of the preanalysis sample treatment (MolYsis Basic) with a complementary matching DNA extraction protocol.

3. Results and discussion

The detection limit of all DNA extraction procedures was determined and is presented in Table 1. Two conventional DNA extraction kits (QIAamp and MPLC) were tested with and without two different preanalysis sample treatment protocols. Preanalysis sample treatment, either with MolYsis Basis or Looxster,

combined with QIAamp extraction, based on a spin-column format, did not result in an increase in detection limit. Both procedures performed equally and were able to detect three target genes at a minimal concentration of 10^3 cfu per ml, which was analogous to 16 cfu per PCR.

Table 1. Lower detection limits of the MRSA real-time PCR assay in relation to preanalysis sample treatment with MolYsis Basic/Looxster and DNA extraction protocol used.

DNA-extraction method	Detection limit[a], no. of cfu/ml							
	10^5	10^4	10^3	$5x10^2$	10^2	$5x10^1$	10^1	10^0
QIAamp	+++	+++	+++	++-	---	---	---	---
QIAamp + M.Basic[b]	+++	+++	+++	+--	---	---	---	---
QIAamp + Looxster[c]	+++	+++	+++	n.d.[d]	---	n.d.	---	---
MPLC	+++	+++	+++	---	---	---	---	---
MPLC + M.Basic	+++	+++	+++	+++	---	---	---	---
MagSi-DNA + M.Basic	+++	+++	+++	+--	---	---	---	---
Complete	+++	+++	+++	+++	+++	+++	++-	---

[a]Detection represented by a Ct-value <40 is indicated by +, no detection is indicated by -, detection was determined for the three gene targets (*mecA*, *femA* and *sa442*). The data represent results from three independent replicate experiments.
[b]MolYsis Basic, preanalysis sample treatment for removal of human DNA and bacteria. enrichment in whole blood samples.
[c] Looxster, preanalysis sample treatment for the enrichment of bacterial DNA from total DNA.
[d] n.d., not determined.

Recently, a study by Horz *et al.* investigated if MolYsis Basic could indeed eliminate human DNA in oral samples to improve the detection of bacterial DNA[8]. They found that use of MolYsis Basic prior to DNA isolation reduced the level of human DNA. However, this effect was accompanied with a partial loss of bacterial DNA. The MPLC kit performed the same as the QIAamp kit, i.e. a detection limit of 10^3 cfu per ml. The MPLC kit in combination with MolYsis Basic could detect 500 cfu per ml. However, when combined with Looxster, the minimal detectable amount of bacteria was 10^4 cfu per ml (data not shown).

This was most likely due to incompatibility of the Looxster Kit and the MPLC elution buffer, which is essential to the performance of the MPLC kit.
The MagSi-DNA kit and the Complete kit represented two novel procedures for targeted isolation of bacterial DNA (Table 1). The MagSi-DNA kit, a novel combination of two sample preparation methods, showed results similar to the results obtained after conventional DNA extraction. The minimal detectable amount of bacteria was 10^3 cfu per ml. MolYsis Complete was able to achieve bacterial detection at a concentration of 50 cfu per ml and therefore achieved the lowest detection limit compared to all other DNA extraction methods. In this case, the detection of 50 cfu per ml was analogous to 4 cfu per PCR. MolYsis Complete provides a combination of preanalysis sample treatment and targeted DNA extraction containing all the buffers and reagents necessary for human DNA removal, bacteria enrichment and bacterial DNA extraction. These findings suggest that the combination of two complementary matching sample preparation procedures and the use of a larger volume of blood sample both contributed to a higher level of bacterial detection. Few studies in the past have focused on pathogen detection in whole blood samples, instead experiments were performed using bacterial suspensions or clinical sample materials such as pleural fluid, pus, synovial fluid and pericardial fluid[9, 10]. Zucol *et al.* evaluated different DNA extraction protocols for whole blood samples followed by broad-range real-time PCR, targeting the 16S rDNA gene in *Staphylococcus aureus* and *Escherichia coli.* They achieved the detection of bacterial concentrations of >10 cfu per PCR, which was analogous to 10^3 cfu per ml[11].
Automation, ease of use, duration and costs of the procedure are each important factors also contributing to the extent of implementation in diagnostic laboratories. Table 2 shows the detection limit, the duration in time and the cost per sample obtained for the different DNA isolation protocols. Except for the MPLC Kit, which is performed on the automated MagNA Pure Instrument, all procedures are performed manually. The extraction methods were all considered as easy to perform. The hands-on time for the manual DNA isolation methods combined with sample pre-treatment varied between 120 and 240 minutes.
In conclusion, we investigated whether the addition of a preanalysis sample treatment could improve the efficiency of purifying bacterial DNA from whole blood samples. The combination of performing a preanalysis sample treatment and using a larger sample volume achieved the detection of only 50 cfu per ml of whole blood (<5 cfu per reaction), emphasizing that the rate of efficiency was attributed to more than one factor. These results confirmed that the efficiency of DNA extraction, especially for clinical samples such as whole blood, is a crucial element in the process of molecular pathogen detection. Ultimately, a combination of optimal sample processing and molecular detection techniques

will lead to rapid and accurate pathogen detection for diagnosis of bloodstream infections.

Table 2. Comparative analysis of the different DNA extraction procedures performed on whole blood spiked with a 10-fold dilution series of MRSA.

DNA extraction method	V_b[a] (ml)	Detection limit (cfu/ml)	Cost[b] (€)	Time[c] (min)
QIAamp	0.2	10^3	2.56	90
QIAamp + M.Basic[d]	0.2	10^3	7.06	175
QIAamp + Looxster[e]	0.2	10^3	32.56	240
MPLC	0.1	10^3	2.27	40
MPLC + M.Basic	0.2	$5x10^2$	6.77	125
MagSi-DNA + M.Basic	0.2	10^3	n.a.[f]	130
Complete	1.0	$5x10^1$	9.70	120

[a] Blood volume in ml.
[b] Price per sample for reagents, not included are plastic wares not provided in the kit.
[c] Hands-on time for eight samples.
[d] MolYsis Basic, preanalysis sample treatment for removal of human DNA and bacteria enrichment in whole blood samples.
[e] Looxster, preanalysis sample treatment for the enrichment of bacterial DNA from total DNA.
[f] n.a., not available.

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Chapter 2b

Pathogen diagnostics for bloodstream infections: bacterial load issues

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Manuscript in preparation

Abstract

In pediatric whole blood specimens, quantification of the bacterial concentration was determined to gain more insights into the requirements needed for pathogen diagnostics. In 46% of the positive blood cultures, colony counts between 20 and 760 colony-forming units (cfu) per ml of whole blood were found. In the remaining samples, no bacteria could be detected without prior enrichment.

1. Introduction

Molecular diagnostics have proven to be successful in the detection of bacterial and viral targets present in high-load patient samples such as cultured materials and faecal samples. However, patient material containing a very low amount of bacteria such as whole blood cannot be analyzed using this rapid and high-throughput approach. As a consequence, whole blood needs culturing, causing a delay of approximately 1-2 days. Methods have been proposed to improve identification of pathogens from whole blood and these are mostly based on the elimination of human DNA and the enrichment of bacterial DNA. So far, only a limited number of studies have been done concerning the bacterial concentration in blood of septic patients, and these have shown contrasting results. In adults, studies have shown that patients contained between 1 and 100 cfu/ml of blood[1]. Furthermore, it is commonly thought that the amount of bacteria is much higher in children, especially in neonates[1]. This, however, can be debated, since reports by Kellogg *et al.* showed the occurrence of low-level bacteraemia (≤10 cfu/ml) in 60-70% of their population[2]. In this study, we wanted to investigate the bacterial load present in children, which could be useful for the determination of diagnostic test requirements in this patient population.

2. Material and methods

In total, we collected 61 pediatric whole blood samples from the department of Pediatrics at the Maastricht University Medical Center (MUMC+, Maastricht, The Netherlands). Whole blood specimens drawn from children suspected of having a bloodstream infection were included in the study if microbial growth was detected in the corresponding blood culture. Pediatric blood culture bottles (BACTEC Peds Plus™, BD Diagnostic Systems) were incubated and monitored for microbial growth in the Bactec 9240 automated blood culture device (BD Diagnostic Systems). Standard conventional testing was performed for bacterial identification. Because of the small amount (<1 ml) of whole blood available from children, 50 µl of whole blood was used for direct inoculation on Columbia sheep blood agar plates (BD Diagnostic Systems). After overnight incubation, colony counting was performed for the determination of the bacterial load.

3. Results

In thirteen blood cultures, which were simultaneously drawn, pathogens were found, whereas 79% of the blood cultures were negative for bacteria. In six of

thirteen cases (46%), we could also find bacteria in the whole blood sample (Table 1).

Table 1. Bacterial load determined in whole blood samples of children with a positive blood culture.

blood culture *positive* whole blood *positive*		blood culture *positive* whole blood *negative*	
Bacterial load in whole blood	Pathogen	Bacterial load in whole blood	Pathogen
20 cfu/ml	CoNS	<20 cfu/ml	CoNS
20 cfu/ml	CoNS	<20 cfu/ml	CoNS
40 cfu/ml	*S. aureus*	<20 cfu/ml	CoNS
20 cfu/ml	*S. aureus*	<20 cfu/ml	CoNS
760 cfu/ml	CoNS	<20 cfu/ml	CoNS
420 cfu/ml	CoNS	<20 cfu/ml	Non-fermenter
		<20 cfu/ml	*Bacillus cereus*

CoNS, coagulase-negative *Staphylococcus* species

The whole blood specimens positive for bacteria showed colony counts between 20 and 760 cfu/ml. Colony counts of <20 cfu/ml could not be detected since this corresponded with less than one colony inoculated from 50 µl of whole blood. This was the case in seven whole blood specimens in which no bacteria were grown, suggesting low-level bacteraemia, not detectable without enrichment. The pathogens found to be present in the blood cultures were *Staphyloccocus aureus* (n=2), coagulase-negative *Staphylococcus* species (CoNS) (n=9), one *Bacillus cereus* and one non-fermenter.

4. Discussion

These findings pointed out the diversity in bacterial load in children suspicious of having a bloodstream infection. Only two out of thirteen whole blood specimens that matched with a positive blood culture contained more than 50 cfu/ml. In the first section of this chapter, we discussed the performance capacities of different DNA extraction protocols and evaluated novel tools for the specific enrichment of bacterial DNA. The detection limit of the most optimal DNA extraction protocol combined with a multiplex real-time PCR was 50 cfu/ml. From that perspective, the molecular detection of pathogens would only be accomplished in two out of thirteen whole blood specimens. The remaining specimens would be missed since less than 50 cfu/ml were present. Jordan *et al.* developed a real-time PCR assay based on 16S rDNA targeting for the detection of bacterial DNA directly from neonatal whole blood. In their study,

spiking experiments were performed to determine the limit of detection. The assay, which used a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) for the bacterial DNA extraction, was capable of detecting 40, 50, or 2000 cfu/ml of *Escherichia coli*, group B *Streptococcus*, and *Listeria monocytogenes*, respectively[3]. Next, the assay was evaluated using a cohort of 53 neonates with culture-proven sepsis, and only two samples failed to be detected[3]. However, it can be questioned to what extent the PCR signal is related to the amount of dead or living bacterial cells, especially in cases of culture-proven sepsis, and in patients receiving antibiotics. Instead, the samples we used for the quantification of the bacterial load were drawn at disease onset from children with unexplained fever, and this was done at admission, before antibiotics were given. Differences in bacterial load can be attributable to several factors such as severity of disease and the pathogen involved. Another report on the diagnosis of bacterial neonatal sepsis found a higher positivity rate with real-time PCR than with blood culture[4]. This was also the case in a recent report presented by Lucignano *et al.* in which they evaluated a multiplex PCR (the LightCycler SeptiFast test, Roche Diagnostics, Mannheim, Germany) for the direct detection of bacteria and fungi from pediatric blood samples. The analytical sensitivity of this test, as indicated by the manufacturer, was between 3 and 100 cfu/ml, depending on the pathogen involved[5]. To clarify the contribution of dead cells, future studies would have to include colony counts of living bacterial cells from whole blood samples, and this in parallel with molecular analysis of the samples. Evaluation of diverse real-time PCR assays showed potential to become a more rapid approach of pathogen diagnostics, offering results within a few hours. Though, it remains to be established whether detection of low-level bacteremia can be achieved. For that reason, blood cultures still remain the diagnostic tool of choice for the detection of bacterial infections.

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Chapter 3a

Molecular probes for the diagnosis of clinically relevant bacterial infections in blood cultures

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Abstract

Broad-range real-time PCR and sequencing of the 16S rDNA gene region is a widely known method for the detection and identification of bacteria in clinical samples. However, because of the need for sequencing, such identification of bacteria is time-consuming. The aim of our study was to develop a more rapid 16S real-time PCR-based identification assay using species or genus-specific probes. The Gram-negative bacteria were divided in *Pseudomonas* spp., *Pseudomonasaeruginosa,Escherichia coli* and other Gram-negatives. Within the Gram-positives, probes were designed for *Staphylococcus* spp., *Staphylococcusaureus*, *Enterococcus* spp., *Streptococcus* spp., and *Streptococcuspneumoniae*. The assay also included a universal probe within the 16S rDNA gene for the detection of all bacterial DNA. The assay was evaluated on a collection of 248 blood cultures. In this study, the universal probe and the probes targeting *Pseudomonas* spp., *P.aeruginosa*, *E. coli*, *Streptococcus* spp., *S.pneumoniae*, *Enterococcus* spp., *and Staphylococcus* spp. all had a sensitivity and specificity of 100%. The probe for *S. aureus* showed eight discrepancies resulting in a sensitivity of 100% and a specificity of 93%. This data showed a high agreement between conventional testing and our novel real-time PCR assay. Furthermore, this assay significantly reduced the time needed for identification. In conclusion, using pathogen-specific probes offers a faster alternative for pathogen detection and could improve the diagnosis of bloodstream infections.

1. Introduction

Bloodstream infections (BSIs) are a major cause of death in the world and need a thorough and adequate therapeutic strategy. Inadequate antibiotic therapy is associated with higher mortality rates, the appearance of antibiotic resistance and longer hospitalisation length of stay[1]. Conventional identification and susceptibility testing have several limitations, such as lack of rapidity and sensitivity. The current gold standard, i.e. blood culture, usually requires 6-12 hours of incubation before growth is detected and a further 24-48 hours for the definitive identification of the infectious agent and its susceptibility to antibiotics[2, 3]. Routine diagnostics already use molecular techniques for the direct detection of viral and bacterial pathogens. However, most in-house assays are targeted against one specific bacterium and/or virus and do not offer a broad-range pathogen detection. Recently, several PCR assays have been developed targeting a panel of the most relevant bacterial and fungal bloodstream pathogens, which can be performed directly from blood, such as Septi*Fast* (Roche Diagnostics, Mannheim, Germany), SepsiTest (MolZym, Bremen, Germany), and VYOO (SIRS-Lab, Jena, Germany), or from positive blood cultures, such as the microarray-based system Prove-itSepsis (Mobidiag, Helsinki, Finland).

As discussed in our previous work[4], direct detection in whole blood is hampered by several factors such as the presence of PCR inhibitors and background DNA, low bacterial load, insufficient sensitivity and the difficulty of establishing an assay capable of detecting a wide range of pathogens. In contrast, molecular testing of growth-positive blood cultures do not require highly sensitive assays because of the presence of a high bacterial load. Furthermore, until now culturing remains essential to determine the microorganism's antimicrobial profile. Therefore, the role of blood cultures remains important for the detection and identification of bacterial causative agents. Molecular testing of blood cultures, possibly in combination with conventional testing, could enable a more rapid identification, and consequently a more rapid diagnosis and start of correct therapy. Molecular approaches such as broad-range real-time PCR and sequencing of the 16S rDNA gene region are widely known methods for the detection and identification of bacteria in clinical samples[5-8]. However, because of the need for sequencing, the identification of bacteria is time-consuming.

The aim of our study was to develop a more rapid 16S real-time PCR-based identification assay using species or genus-specific probes. The assay is particularly intended for identification of positive blood cultures, of which Gram staining results are known. In this proof-of-concept study, priority was given to the genera or species most frequently found in blood cultures and/or those that could direct the choice of a suitable antibiotic therapy. Therefore, we selected a panel of eight species or genus-specific probes. The Gram-negative bacteria

were divided into *Pseudomonas* spp., *P. aeruginosa, E. col*i and other Gram-negatives. Within the Gram-positives probes were designed for *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp., *Streptococcus* spp., and *S. pneumoniae*. Hence, a first indication about the causative microorganism is given after two hours, while confirmation and precise identification can be achieved with sequencing. Consequently, multiple species can be detected in samples with a polymicrobial infection. The present paper reports a retrospective study performed on blood cultures obtained from patients with suspected bloodstream infections. Results of this new multi-probe assay were compared with conventional blood culture findings.

2. Material and methods

Clinical samples

A total of 248 blood cultures were collected at the Maastricht University Medical Center (MUMC+, Maastricht, The Netherlands). All samples were analyzed with standard conventional testing. Blood drawn from patients suspected for bloodstream infection was incubated in blood culture bottles (Plus+Aerobic (product no. 442192;BD Diagnostic Systems, Sparks, MD, USA) and Plus+Anaerobic (product no. 442193; BD) and monitored for microbial growth in the Bactec™ 9240 automated blood culture device (BD Diagnostic Systems). When growth was detected, Gram-staining was performed. A small aliquot of each blood culture (1 ml) was requested for the novel molecular assay. Two separate assays were developed for Gram-negative and Gram-positive bacteria, respectively. Hence, further analysis was based on the results of Gram-staining.

Conventional bacterial identification

Regarding the Gram-positive cocci: to discern *Staphylococcus* spp. from other Gram-positive cocci (GPCs), a catalase test was performed by adding one colony to a drop of 3% H_2O_2. For the identification of *Staphylococcus* spp., catalase-positive strains were tested for coagulase and DNase production. If both tests were negative, the strain was identified as a coagulase-negative *Staphylococcus* (CoNS). To discern *Enterococcus* spp. from other catalase-negative GPCs, bile esculin, tellur diagnostic tablets (Rosco Diagnostica, Taastrup, Denmark) and an API 20 Strep test (Biomérieux SA, Marcy l'Etoile, France) were used, according to manufacturer's guidelines. Optochin susceptibility (OXOID, Hampshire, United Kingdom) was used to differentiate *S. pneumoniae* from the other viridans streptococci, which were further identified by API 20 Strep. In case of β-hemolytic streptococci, latex agglutination was

performed using the Prolex Streptococcal Grouping Latex kit (Bio Trading, product code PL.030).

Bacterial strains

Reference strains were used to validate the specificity of the assay, including *S. aureus* ATCC 25923 and 29213, *Staphylococcus epidermidis* ATCC 12228 and 14990, *S. pneumoniae* ATCC 49619, *P. aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* BM 4147, and *E. coli* ATCC 35218.

Multi-probe assay

An aliquot (0.1 ml) of blood culture was 1:100 diluted in 0.9% NaCl. Dilutions were centrifuged for 5 min at 12.000 rpm and the bacterial pellet was resuspended in 100 µl NASBA H_2O. The primers and the universal bacterial TaqMan probe have been described previously [9]. The probes for *P. aeruginosa*, *Pseudomonas* spp., *E. coli*, *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp., *Streptococcus* spp., and *S. pneumoniae* were designed by using the BLAST tool and ClustalW software. Multiple sequence alignments were made and are partly shown in Figure 1. The designed probe sequences are given in Table 1. All primers and probes were tested for specificity and cross-reactions both manually and with use of the NCBI-BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). Primer and probe matrices were performed to determine optimal concentrations. Each test contained 5 µl purified sample and 20 µl reaction mixture. The reaction mixture contained 12.5 µl of Taqman Environmental Master Mix 2.0 (Applied Biosystems, Foster City, California, United States), 0.9 µM of forward primer, 0.6 µM of reverse primer, and 0.2 µM of each probe. There were three separate reactions. The first reaction included the universal probe and *P. aeruginosa* probe. The second reaction contained the *E. coli* and *Pseudomonas* spp. probe. The third reaction included the *Staphylococcus* spp. probe, the *S. aureus* probe and the *Enterococcus* spp. probe, the third and final reaction the *Streptococcus* spp. probe and the *S. pneumoniae* probe. Reactions were performed on the ABI PRISM7000 real-time PCR System (Applied Biosystems, Foster City, California, United States) and optimal thermal cycling conditions were as follows: 2 min at 50°C, initial denaturation at 95°C for 15 min, 42 cycles of denaturation for 15 s at 95°C and annealing at 60°C for 1 min. Cycle threshold (Ct), the cycle number at which amplicon fluorescence exceeded the preset detection threshold, was recorded for all samples. The cut-off value to consider a PCR result as positive was set to a Ct of 35.

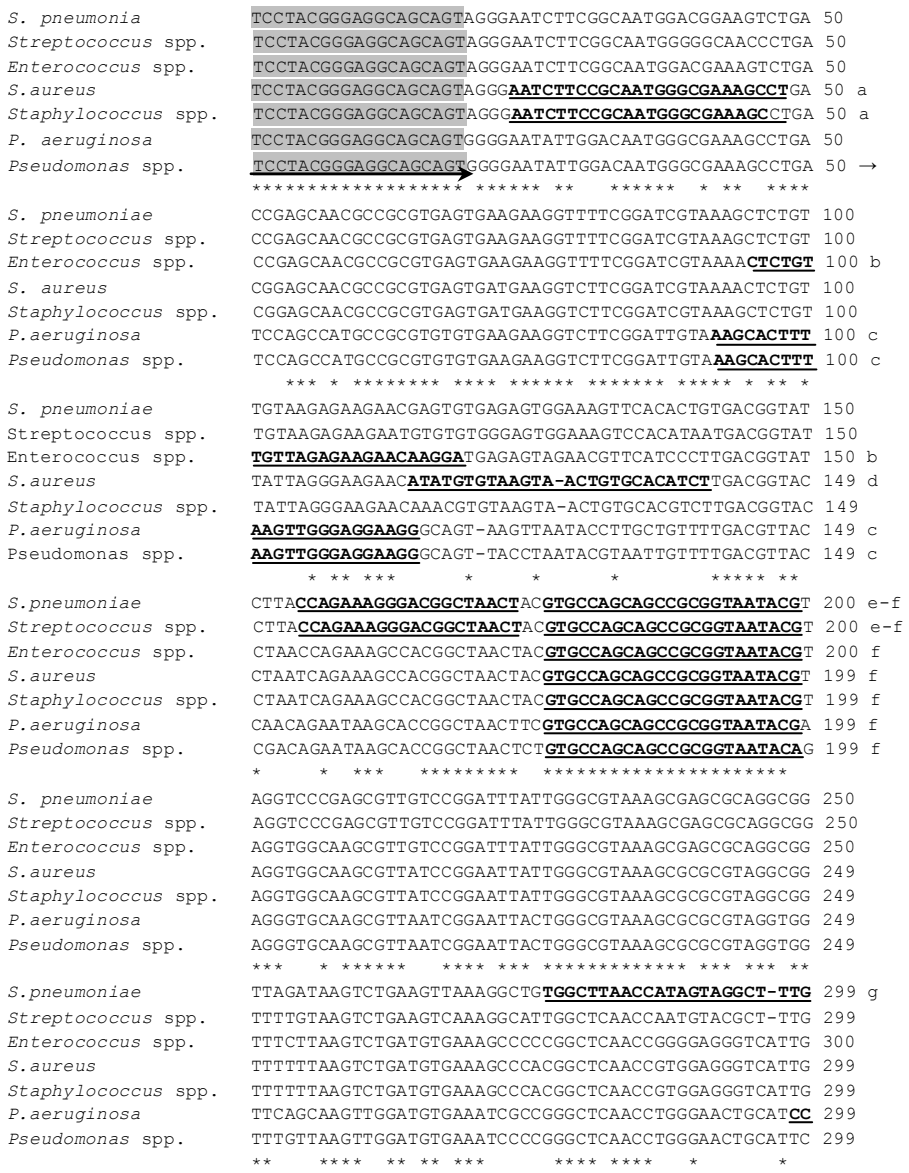

Figure 1. Multiple sequence alignments of the amplified part of the bacterial 16S gene. Sequences are shown of the bacteria included in the multi-probe assay. The arrows (←→) represent the forward and reverse primer targeting the 16S gene. The sequences of the probes are in bold and underlined: (a) *Staphylococcus* spp. probe. (b) *Enterococcus* spp. probe. (c) *Pseudomonas* spp. probe. (d) *S. aureus* probe. (e) *Streptococcus* spp. probe. (f) Universal probe. (g) *S. pneumoniae* probe. (h) *P. aeruginosa* probe.

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S.pneumoniae          GAAACTGTTTAACTTGAGTGCAAGAGGGGAGAGTGGAAT-CCATGTGTAG 348
Streptococcus spp.    GAAACTGCGAGACTTGAGTGCAGAAGGGGAGAGTGGAATTCCATGTGTAG 349
Enterococcus spp.     GAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAG 350
S.aureus              GAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAG 349
Staphylococcus spp.   GAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAG 349
P.aeruginosa          AAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATT-CCTGTGTAG 348 h
Pseudomonas spp.      AAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAG 349
                       *****      ** ****     ** **   *******  * *******
S.pneumoniae          CGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCT 398
Streptococcus spp.    CGGTGAAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCT 399
Enterococcus spp.     CGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCT 400
S.aureus              CGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTT 399
Staphylococcus spp.   CGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGGCTTT 399
P.aeruginosa          CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCAC 398
Pseudomonas spp.      CGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCAC 399
                      ************ *** *** * ********* ******** *** *
S.pneumoniae          CTGGCTTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGAT 448
Streptococcus spp.    CTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGAT 449
Enterococcus spp.     CTGGTCTGTAACTGACGCTGAGGCTCGAAAGCGTGGGGAGCGAACAGGAT 450
S.aureus              CTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGAT 449
Staphylococcus spp.   CTGGTCTGCAACTGACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGAT 449
P.aeruginosa          CTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 448
Pseudomonas spp.      CTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT 449 ←
                      ****  *   ****** **** *  ************ * * ********
S.pneumoniae          TAGATACCCTGGTAGTCC 466
Streptococcus spp.    TAGATACCCTGGTAGTCC 467
Enterococcus spp.     TAGATACCCTGGTAGTCC 468
S.aureus              TAGATACCCTGGTAGTCC 467
Staphylococcus spp.   TAGATACCCTGGTAGTCC 467
P.aeruginosa          TAGATACCCTGGTAGTCC 466
Pseudomonas spp.      TAGATACCCTGGTAGTCC 467 ←
                      ******************
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Figure 1.-Continued.

Table 1. Probes designed for use in multiplex PCR

Probe	Sequence (5'-3')[a]
Pseudomonas spp.	NED-CCTTCCTCCCAACTTAAAGTGCTT-MGB
P. aeruginosa	JOE-CCAAAACTACTGAGCTAGAGTACG-BHQ1
E. coli	JOE-GGAGTAAAGTTAATACCTTTGCTCATT-BHQ1
Staphylococcus spp.	NED-AATCTTCCGCAATGGGCGAAAGC-MGB
S. aureus	FAM-AGATGTGCACAGTTACTTACACATAT-BHQ1
Enterococcus spp.	JOE-TCCTTGTTCTTCTCTAACAACAGAG-BHQ1
Streptococcus spp.	NED-CCAGAAAGGGACSGCTAACT-MGB
S. pneumoniae	JOE-CCAAAGCCTACTATGGTTAAGCCA-BHQ1

[a]NED, fluorescent label (Applied Biosystems, (Foster City, CA, USA); MGB, minor groove binder; JOE, 6-carboxy-4,5-dichloro-2,7-dimethyoxyfluorescein; BHQ1, black hole quencher 1;FAM, 6-carboxyfluorescein.

DNA sequencing

Samples with discrepant results were further analyzed with sequencing. The DNA was purified using the MSB® PCRapace PCR Cleanup Kit (Invitek, Berlin, Germany) and dissolved in 30 µl NASBA H_2O. Cyclic sequencing was performed using BigDye 3.0 (Applied Biosystems, Foster City, California, United States). The resulting sequence product was purified and separated on the 3730 DNA Analyzer (Applied Biosystems, Foster City, California, United States).

3. Results

Design of the species or genus-specific probes

The specificity of the probes was evaluated using BLAST. In silico analysis revealed that nearly only cross-reactivity was found with not clinically relevant microorganisms such as *Geobacillus* spp. and *Lactobacillus plantarum.* In silico cross-reactivity with clinically relevant microorganisms was tested in vitro with reference strains. For example, in silico the *E. coli* probe cross-reacted with *Acinetobacter* spp. and *Enterobacter* spp. However, in vitro tests showed no positive signals for these microorganisms. Furthermore, cross-reactions were found with *Escherichia albertii* and *Shigella* spp. Based on literature, these microorganisms only occur rarely in blood cultures and therefore the risk of false identification was minimal. In a few cases such as with the *Streptococcus* spp. and the *Staphylococcus* spp. probe, cross-reactivity was found with *Bacillus cereus*. The species conferring cross-reactivity in PCR were then sequenced to check for mismatches between the probe-target hybrids. Sequencing results showed that this occurred because of at least two mismatches at the binding site of both probes (data not shown). However, in all these cases the cross-reacting fluorescent signals generated by the mismatched probes were weak compared to the positive template control and uncharacteristically appeared over 5 cycles later than signals from 100% matched probes (data not shown).

Evaluation of the multi-probe assay by testing clinical blood culture samples

From a total of 248 blood cultures, the presence or absence of bacterial DNA was determined in 232 growth-positive and 16 growth-negative samples using the universal probe, yielding a sensitivity and specificity of 100% (Table 2).

Table 2. Bacterial isolates in 232 positive blood cultures and results from universal and specific real-time PCR.

	Result of blood culture (no. [%])[a]							
Pathogen	**uni**	**pseu**	**pseuae**	**ecoli**	**staph**	**stau**	**ente**	**strept**	**strepn**
Gram-negative pathogens:									
Acinetobacter lwoffii/haemolyticus	1 (100)	0	0	0	-	-	-	-	-
Bacteroides fragilis	2 (100)	0	0	0	-	-	-	-	-
Citrobacter koseri	1 (100)	0	0	0	-	-	-	-	-
Enterobacter cloacae	3 (100)	0	0	0	-	-	-	-	-
Escherichia coli	43 (100)	0	0	43	-	-	-	-	-
Gram-negative rod	2 (100)	0	0	0	-	-	-	-	-
Klebsiella oxytoca	5 (100)	0	0	0	-	-	-	-	-
Klebsiella pneumoniae	7 (100)	0	0	0	-	-	-	-	-
Moraxella catarrhalis	1 (100)	0	0	0	-	-	-	-	-
Morganella morganii	1 (100)	0	0	0	-	-	-	-	-
Neisseria meningitidis	1 (100)	0	0	0	-	-	-	-	-
Prevotella buccae	1 (100)	0	0	0	-	-	-	-	-
Proteus mirabilis	2 (100)	0	0	0	-	-	-	-	-
Pseudomonas aeruginosa	10 (100)	10	10	0	-	-	-	-	-
Pseudomonas oryzihabitans	1 (100)	1	0	0	-	-	-	-	-
Serratia marcescens	4 (100)	0	0	0	-	-	-	-	-
Gram-positive pathogens:									
Aerococcus viridans	1 (100)	-	-	-	0	0	**1**	0	0
Coag.-neg.*Staphylococcus* spp.	85 (100)	-	-	-	85	**9**	0	0	0
Corynebacterium spp.	2 (100)	-	-	-	0	0	0	0	0
Enterococcus avium	1 (100)	-	-	-	0	0	1	0	0
Enterococcus faecalis	3 (100)	-	-	-	0	0	3	0	0
Enterococcus faecium	4 (100)	-	-	-	0	0	4	0	0
Lactobacillus spp.	1 (100)	-	-	-	0	0	0	0	0
Propionibacterium acnes	1 (100)	-	-	-	0	0	0	0	0
Staphylococcus aureus	31 (100)	-	-	-	31	31	0	0	0
Streptococcus agalactiae	1 (100)	-	-	-	0	0	0	1	0
Streptococcus group A and/or D	2 (100)	-	-	-	0	0	0	2	0
Streptococcus milleri	3 (100)	-	-	-	0	0	0	3	0
Streptococcus oralis	1 (100)	-	-	-	0	0	0	1	0
Streptococcus pneumoniae	6 (100)	-	-	-	0	0	0	6	6
Streptococcus pyogenes	1 (100)	-	-	-	0	0	0	1	0
Streptococcus sanguis	1 (100)	-	-	-	0	0	0	1	0
Mixed:									
Alcaligenes faecalis, Propionibacterium spp.	1 (100)	0	0	0	0	0	0	0	0
Klebsiella oxytoca, *Serratia marcescens*	1 (100)	0	0	0	-	-	-	-	-
Streptococcus salivarius, *Streptococcus viridans*	1 (100)	-	-	-	0	0	0	1	0
Totals:									
Concordant		11	10	43	116	31	8	16	6
Discordant		0	0	0	0	9	1	0	0

[a] uni, universal probe; pseu, *Pseudomonas* spp.; pseuae, *P. aeruginosa*; ecoli, E. coli; staph, *Staphylococcus* spp.; stau, *S. aureus*; ente, *Enterococcus* spp.; strept, *Streptococcus* spp.; strepn, *S. pneumoniae*

Our multi-probe assay was performed after Gram-staining of the positive blood cultures. Considering the growth-positive blood cultures, results from the multi-probe assay were in accordance with conventional identification in 222 (96%) cases. The specific probes targeting *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Streptococcus* spp., *S. pneumoniae,* and *Staphylococcus* spp. all had a sensitivity and specificity of 100%. The majority, 85 of 145 (59%) Gram-positive blood cultures contained a CoNS. In thirty-one blood cultures, the causative agent was identified as *S. aureus*. Regarding the Gram-positive staphylococcal blood cultures, the multi-probe assay was in conflict with culture results in nine cases. The nine discrepancies were further analyzed by coagulase testing, specific *S. aureus* real-time PCR, and sequencing (Table 3, Figure 2). One out of nine cases, clinical isolate (CI) 7, was tested coagulase-positive, and was positive for two targets specific for *S. aureus,* i.e. *femA* and *sa442*. Sequencing of the PCR product also confirmed the results of the multi-probe assay. The sequences of the eight other clinical isolates showed three mismatches compared with the reference *S. aureus* sequence, as shown in Figure 2A. Hence, the remaining eight cases were confirmed as discordant. Consequently, a sensitivity and specificity of 100% and 93%, respectively, and a positive and negative predictive value of 79% and 100%, respectively was achieved when sequencing was considered as gold standard. The probe specific for *Enterococcus* spp. showed one conflicting result. One blood culture was determined to contain an *Aerococcus viridans*, while our multi-probe assay identified the infectious agent as *Enterococcus* spp. (Table 3).

Table 3. Clinical isolates with discordant blood culture and PCR results

Blood culture	**Specific probes** [b]	**femA, sa442** [c]	**Coagulase activity**	**Sequence**
CI[a] 1: CoNS	staph, stau	N-N	N	*Unidentified*
CI 2: CoNS	staph, stau	N-N	N	*S. hominis*
CI 3: CoNS	staph, stau	N-N	N	*S. hominis*
CI 4: CoNS	staph, stau	N-N	N	*S. pasteuri*
CI 5: CoNS	staph, stau	N-N	N	*S. hominis*
CI 6: CoNS	staph, stau	N-N	N	*S. schleiferi*
CI 7: CoNS	staph, stau	P-P	P	*S. aureus*
CI 8: CoNS	staph, stau	N-N	N	*S. pasteuri*
CI 9: CoNS	staph, stau	N-N	N	*S. epidermidis*
CI 10: *A. viridans*	ente	n.a.	n.a.	*Enterococcus* spp.

[a] CI, clinical isolate

[b] staph, *Staphylococcus* spp. ; stau, *S. aureus* ; ente, *Enterococcus* spp.

[c] N, negative ; P, positive; n.a., not applicable

Further analysis revealed that the partial sequence of CI 10 was completely similar to the sequence of a reference *Enterococcus* spp. strain, as shown in Figure 2B. Comparison of reference *A. viridans* and *Enterococcus* spp. strains

showed five mismatches. Consequently, the probe specific for *Enterococcus* spp. yielded a sensitivity and specificity of 100% when sequencing was considered as gold standard.

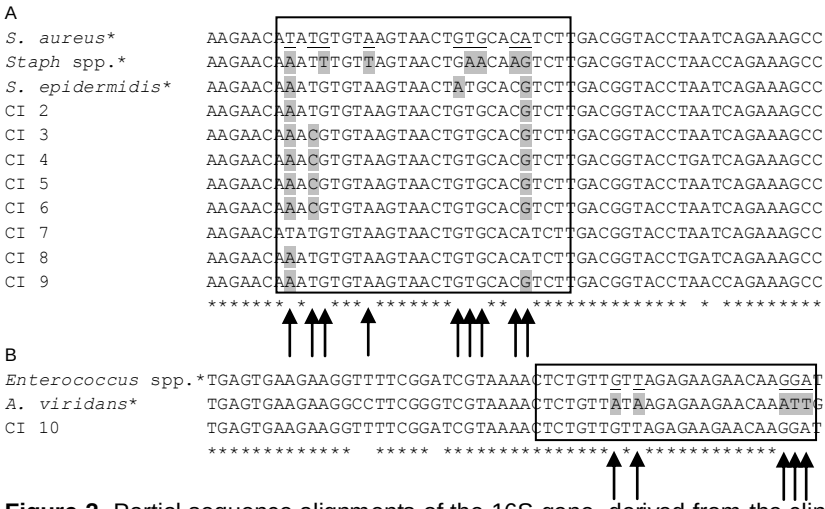

Figure 2. Partial sequence alignments of the 16S gene, derived from the clinical isolates with discordant results. A) i.e. region where the probe specific for *S. aureus* binds. The sequences of the eight discrepancies are shown in the rectangle. The nucleotides underlined in the rectangle represent the nucleotides which distinguish a *S. aureus* from a *Staphylococcus* spp. The sequence alignment of clinical isolate (CI) 7 is the exact reverse complement of the *S. aureus* probe sequence, which implicates that this isolate, which was identified as CoNS, is in fact a *S. aureus*. CI 1 was not shown because the sequence could not be determined. Accession numbers reference strains: *S. aureus* FN433596, *Staphylococcus* spp. GQ222246, *S. epidermidis* GQ911565. B) i.e. region where the probe specific for *Enterococcus* spp. binds. The nucleotides underlined in the rectangle represent the nucleotides which distinguish an *Enterococcus* spp. from an *A. viridans*. Conventional testing identified CI 9 as *Aerococcus viridans*, whereas our assay and sequencing classified it as *Enterococcus* spp. Accession numbers reference strains: *Enterococcus* spp. AB489105, *A. viridans* M58797. *, reference sequences; CI, clinical isolate; CoNS, coagulase-negative *Staphylococcus* spp.

4. Discussion

New strategies for the detection and identification of bacterial pathogens in blood are continuously under investigation worldwide. Current diagnostic tools are hampered by several factors such as a lack of sensitivity and a long time-to-results, the presence of PCR inhibitory compounds in certain sample materials, and the occurrence of fastidious and non-culturable microorganisms. Rapid universal 16S rDNA gene-based PCR is often combined with sequence analysis, and a considerable number of commercial assays have been

generated and tested recently, i.e. Septi*Fast* (Roche Diagnostics, Mannheim, Germany), Prove-itSepsis (Mobidiag, Helsinki, Finland), and SeptiTest (MolZym, Bremen, Germany). The latter includes a universal 16S rDNA gene-based PCR assay combined with sequence analysis, while the two other commercial assays use a panel of probes for the detection of a range of bacterial and mycotic pathogens. Virtually all studies conducted with the Septi*Fast* and SeptiTest assays found similar results, i.e. an overall agreement of 77-83%[5, 10-15]. Both assays are validated for whole blood samples and can be completed in four to six hours. Tissari *et al.* performed an observational study comparing conventional culture with Prove-it™ Sepsis. The assay, based on a microarray platform and performed on blood cultures, had a clinical sensitivity of 94.7% and a specificity of 98.8%[16].

In our study, the 16S rDNA gene was used for the design of eight species or genus-specific probes. The sequence similarities found in in silico analysis (BLAST) were mainly derived from not clinically relevant microorganisms such as *Geobacillus* spp., *Paenibacillus* spp., *L. plantarum*, and *L. fermentum*. Remaining similarities were, if available, tested *in vitro* with reference strains. A limited number of microorganisms showed cross-reactivity. For example, streptococcal and staphylococcal probes weakly cross-reacted with *B. cereus*. In these cases, sequencing determined the correct identification. Additionally, as the assay is always preceded by Gram-staining, in most cases morphology data from Gram-staining was in contradiction with these false-positive signals and targeted the samples for confirmatory sequencing.

Our multi-probe assay was designed for use on blood culture material and can be completed within two hours. Use of whole blood samples could significantly improve the turnaround time of our assay. However, since culturing still remains essential to determine the micro-organisms antimicrobial profile, we chose to use blood cultures instead of whole blood. In this study, we found an overall agreement of 97% between conventional testing and our multi-probe assay. One of the strengths of our approach is that the assay can be extended by adding more probes for other bacterial pathogens to the identification panel. The grouping of Gram-negative and Gram-positive bacteria was based on the identifications needed for antimicrobial susceptibility testing. The assay was designed to use a universal probe for detection, instead of SYBR Green, because it is known that SYBR Green can generate false-positive signals because of the presence of background human DNA or the formation of primer-dimers.

Analysis of the conflicting results showed that in some cases our assay was in accordance with sequencing results. The reference sequence derived from a *Staphylococcus* spp. and a *S. epidermidis* strain showed seven and three mismatches, respectively, compared with the reference sequence derived from a *S. aureus* strain. The sequences derived from the discrepant clinical isolates

only showed three or less mismatches compared with the *S. aureus* sequence. This indicated that within the group of staphylococci more homologous sequences can cause false-positives because of the less efficient binding capacity of the *S. aureus*-specific probe.
Overall, these results showed a strong agreement between conventional testing and our novel, real-time PCR assay. Furthermore, this assay significantly reduced the time needed for identification in comparison to routine diagnostics. In conclusion, using pathogen-specific probes offers a faster alternative for pathogen detection and could improve the diagnosis of bloodstream infections. The assay will be implemented in a clinical trial investigating the impact of earlier pathogen identification in combination with susceptibility testing on the choice of therapy.

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Chapter 3b

Rapid identification of *Candida* species in blood cultures by multiprobe TaqMan PCR assay

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Abstract

We report a multiprobe TaqMan PCR assay for detection and differentiation of four clinically relevant *Candida* species in blood culture samples. We developed and evaluated species specific probes which were used in conjunction with a universal *Candida* probe. All probes targeted the internal transcribed spacer regions and for identification of *Candida albicans*, *Candida parapsilosis*, *Candida glabrata* and *Candida krusei* in two reaction tubes in a single run of real time PCR. The assay can give results in three hours after detection of yeast cells in positive blood culture by Gram staining. DNA detection limits of the assay for *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei* were 15, 50, 350 and 5 colony-forming units (cfu) per ml, respectively.

1. Introduction

There is an increasing patient population at high risk of acquiring opportunistic infections due to a.o. the use of immunosuppressive therapy. Within these immunocompromised patients, invasive fungal infections (IFIs) are more and more recognized as an important cause of morbidity and mortality[1]. Among the fungi the most common causative agents of opportunistic infections are *Candida* species and invasive infections with *Candida* species have significant mortality rates between 38 to 74%[2]. Although *Candida albicans* remains the leading pathogen of IFIs and is the most commonly found Candida species in blood cultures, there are many reports that point out the increase in incidence of non-albicans *Candida* species such as *Candida glabrata* and *Candida parapsilosis*[3-5]. Infections with non-albicans *Candida* species are important because of their resistance profiles to antifungal agents. *C. glabrata* and *Candida krusei* have reduced susceptibility to azoles which are the most frequently used antifungal agents in clinical practice. *C. parapsilosis* displays decreased susceptibility to echinocandins. Thus, rapid and accurate identification of fungal species guides the early initiation of appropriate antifungal therapy and is therefore of crucial importance for patient outcome[6].
Blood culture is accepted as a gold standard for diagnosis of IFIs. Identification of *Candida* species in positive blood cultures with conventional biochemical and phenotypic methods requires commonly two or three days. Therefore, the availability of a rapid test for identification of *Candida* spp., more specifically *C. glabrata* and *C. krusei* could prevent delays in the administration of appropriate therapy[7, 8]. In recent years, different molecular approaches have been developed for rapid identification of *Candida* species from blood or blood cultures[8-10]. However, most assays identified only a limited number of *Candida* species and/or did not include pan-*Candida* or panfungal primers or probes to detect those species not specifically targeted.
The aim of our study was development of a rapid assay for identification of four clinically relevant *Candida* species in conjunction with a universal *Candida* probe detecting all commonly found *Candida* species. The assay is intended to be used on positive blood culture samples after the detection of yeast cells by Gram-staining.

2. Materials and Methods

Yeast Strains and Clinical Samples

Clinical fungal isolates collected at the Department of Medical Microbiology of Maastricht University Medical Center (MUMC+, Maastricht, The Netherlands) were cultivated on Sabouraud dextrose agar for 48 hours at 30^{o}C. Dilutions of

yeast cells were prepared with sterile saline suspensions that were adjusted to match that of a 0.5 McFarland standard. Blood culture samples for spiking experiments were obtained from negative blood culture bottles. Whereas for the pilot study blood culture bottles (BACTEC Peds Plus™, BD Diagnostic Systems) were incubated and monitored for microbial growth in the Bactec 9240 automated blood culture device (BD Diagnostic Systems). Standard conventional testing was performed for fungal identification. All clinical samples were anonymized and treated according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies.

DNA Extraction

A total of 200 µl of blood culture sample was washed twice by adding it to 1 ml of sterile demineralized water, and centrifuging it at 13,000 x g for 2 min. The pellet was resuspended in 500 µl of lyticase solution (50 U/ml lyticase (Sigma Aldrich, St. Louis, MO, USA), 50 mM Tris [pH 7.5], 10 mM EDTA, 28 mM β-mercaptoethanol) and incubated 37°C for 30 min to produce spheroplasts. After centrifugation, QIAamp DNA Mini kit (Qiagen, Hilden, Germany) was used and the pellet was suspended in 180 µl Buffer ATL and 20 µl proteinase K and incubated at 56°C for 15 min with occasional vortexing. After incubation 200 µl Buffer AL (provided in the QIAamp DNA mini kit) was added and incubated at 70°C for 10 min. A total of 600 µl water was added followed by 800 µl >99% benzyl alcohol (Merck) and vortexed. In order to separate the phases, the sample was centrifuged at 20,000 x g for 5 min and 200 µl of supernatant was transferred to a QIAamp Mini spin column. The column was centrifuged at 6,000 x g for 1 min. A 500 µl of AW1 buffer was added, and incubation of 5 min at room temperature was followed by centrifugation at 6,000 x g for 1 min. The spin column was placed into a new collection tube, and 500 µl of AW2 buffer was added and centrifuged at 20,000 x g for 3 min. The spin column was placed into a new collection tube and centrifuged at 20,000 x g for 1 min as recommended in manufacturer's protocol. The DNA was extracted from the spin with adding 50 µl of AE buffer. The column was incubated at room temperature for 1 min and centrifuged at 6,000 x g for 1 min.

Primer and TaqMan Probe Design

The universal amplification primers ITS1, ITS2, ITS3[11] and ITS4-rev (designed for this study) were used to amplify ITS regions of rDNA. Uni-*Candida* probe designed by Shin *et al.*[12] was used as a *Candida* genus-specific probe (Table 1). The real-time PCR primer and probes were designed based on *in silico* analysis of the ITS region using BLAST(http://www.ncbi.nlm.nih.gov/BLAST). To find the most conserved regions the sequences were aligned using ClustalW2

software. The sequences of primers and probes were checked for cross-reactions by using the BLAST algorithm. Oligonucleotides with the reporter dyes FAM and JOE were synthesized by Sigma Aldrich, NED-labeled probes were obtained from Applied Biosystems (Foster City, CA, USA).

PCR Conditions

To determine the optimal primer and probe concentrations primer and probe matrices were performed. For each sample, two multiprobe reaction mixtures were used. One of PCR mixtures consisted of 2x Qiagen Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 300 nM concentration of ITS1 and ITS2 primers, 250 nM *C. glabrata* probe, 250 nM *C. parapsilosis* probe and 100 nM *C. krusei* probe. The other PCR mixture consisted of 2× QIAGEN Multiplex PCR Master Mix, 300 nM concentration of ITS3 and ITS4-rev primers, 250 nM Uni-*Candida* probe and 100 nM *C. albicans* probe. DNA samples were analyzed by using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) according to the following cycling parameters: 2 min at 50°C, 15 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 60°C.

Table 1. Primers and probes used for detection and identification of *Candida* species in two reaction mixtures.

Primer/Probe	Sequence	Reference
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	[11]
ITS2	5'-GCTGCGTTCTTCATCGATGC-3'	[11]
C. glabrata	5'-JOE-TGTCTGAGCTCGGAGAGAGACATC-BHQ1-3'	This study
C. krusei	5'-NED-CACTGCGTGAGCGGAACGAAAACA-MGB-NFQ-3'	This study
C. parapsilosis	5'-FAM-TTGGTAGGCCTTCTATATGGGGCCT-BHQ1-3'	This study
ITS3	5'-GCATCGATGAAGAACGCAGC-3'	[11]
ITS4-rev	5'-TATGCTTAAGTTCAGCGGGT-3'	This study
Uni-*Candida*	5'-JOE-AGGGCATGCCTGTTTGAGCGTC-BHQ1-3'	[12]
C. albicans	5'-NED-CATTGTCAAAGCGATCCCGCCTTA-MGB-NFQ-3'	This study

Assay evaluation

The lower limit of detection (LOD) for each *Candida* species was determined by using negative blood culture samples spiked with serially diluted yeast cell suspensions to the final concentrations of 10^6 to 10^0 cfu/ml. *Candida* concentrations were determined by plating 100 µl of the diluted sample onto Sabouraud dextrose agar plate. The probes were evaluated for possible cross-

reactivity in vitro against blood culture samples spiked with various microorganisms, i.e. *Candida tropicalis, Candida lusitanae, Candida dubliniensis* and *Candida guillermondii* as well as *Saccharomyces cerevisiae, Cryptococcus neoformans, Trichosporon* spp., *Verticillium* spp., *Aspergillus fumigatus* and the bacteria *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Streptococcus* spp.

3. Results

Results showed that there was no cross-reactivity between the species-specific probes of our multiprobe assay nor with *C. tropicalis, C. lusitanae, C. dubliniensis* and *C. guillermondii*. In addition, we also observed no false-positive results with the following various fungi, i.e. *Trichosporon* spp., *S. cerevisiae* and *C. neoformans*, as well with the following bacteria which are frequently found in blood cultures, i.e. coagulase-negative staphylococci (CoNS), *S. aureus*, *P. aeruginosa*, *E. coli*, *Klebsiella* spp. and *Streptococcus* spp. However, we did observe cross-reactivity of the universal *Candida* probe with other fungi, i.e. *Verticillium* spp., *S. cerevisiae* and *C. neoformans*.

The assay was combined with an optimized protocol for DNA extraction of *Candida* species from blood culture material. The optimized DNA extraction protocol included modifications on the enzymatic lysis method of the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) procedure and added a phase separation step with benzyl alcohol which allowed us to overcome PCR inhibitors. The complete assay including DNA isolation and multiprobe assay was evaluated using negative blood cultures spiked with grown *Candida* species. The results showed that the limits of detection for *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei* were 15, 50, 350 and 5 cfu/ml, respectively. We found no differences between the samples obtained from aerobic, anaerobic and pediatric bottles with similar inoculums (data not shown).

Finally, in a pilot experiment, five sets of blood cultures in which fungal cells were detected by Gram-staining, were tested by the new assay. Conventional culturing identified one set to be containing *Trichosporon* spp., three sets containing *C. albicans* and one set containing *C. glabrata*. All positive *Candida* cultures were correctly identified, showing both positive signals by the universal *Candida* probe as well as the respective species probe. All real-time PCR signals had Ct values between 18 and 25 by the respective probes. The cultures containing *Trichosporon* spp. correctly showed no positive signal. From one of the sets containing *C. albicans*, additional blood culture bottles from four days earlier were available. All four of these blood culture bottles had remained culture-negative. Upon testing, three out of four bottles showed positive PCR results for *C. albicans* with high Ct values between 33 and 38.

4. Discussion

In this study, we developed and evaluated a multiprobe assay including a universal *Candida* probe and four species-specific probes for the rapid identification of *Candida* species from blood cultures. The first step of the complete assay consisted of an optimized DNA isolation protocol which included enhanced enzymatic lysis and a phase separation step with benzyl alcohol to overcome PCR inhibitors. Blood culture media contains several PCR inhibitors, and one of them is sodium polyanethol sulfonate (SPS). Therefore, several studies included benzyl alcohol separations for the removal of PCR inhibitors from blood culture media[13, 14].

The design of the multiprobe real-time PCR assay included four species-specific probes for the detection of *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei.* While our species-specific probes showed no cross-reactivity with DNA templates isolated from a panel of bacterial and fungal species, the uni-*Candida* probe cross-hybridized with DNA of several other fungal species. Therefore, in our setup a positive signal obtained from the Uni-*Candida* probe alone may not be interpreted as the presence of *Candida* species and should be further evaluated by using DNA sequencing. The analytical sensitivity of the complete multiprobe assay differed per species between 350 and 5 cfu/ml. This is in the same range or slightly higher than other published *Candida* real-time PCR assay data[15, 16]. However, in our study we used aliquots of negative blood culture bottles spiked with yeast cells in contrast to fresh whole blood samples used in most other studies. Our higher detection limits may be related with the presence of inhibitors from blood culture media. Our results presenting low Ct values between 18 and 25 in positive blood cultures showed that the analytical sensitivity of the assay when used on blood cultures is more than sufficient. However, in analogy with earlier work on positive blood cultures containing bacteria, utilizing the assay's sensitivity to its full capacity could decrease the time that is needed for incubation of blood cultures[17]. Future (prospective) studies on clinical samples will have to be performed to establish the clinical sensitivity and specificity as well as to indicate the potential for use on other clinical materials.

In conclusion, the novel multiprobe assay detects *Candida* species and identifies *C. albicans*, *C. parapsilosis*, *C. glabrata* and *C. krusei* in two reaction tubes in a single run and it provides results in three hours after detection of yeast cells by Gram staining of positively flagged blood culture samples. Therefore, this assay allows rapid identification of *Candida* species and may provide an opportunity to start appropriate antifungal treatment in a timely manner in patients with candidemia.

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Chapter 4

A real-time PCR-based semi-quantitative breakpoint to aid in molecular identification of urinary tract infections

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Abstract

This study presents a novel approach to aid in diagnosis of urinary tract infections (UTIs). A real-time PCR assay was used to screen for culture-positive urinary specimens and to identify the causative uropathogen. Semi-quantitative breakpoints were used to screen for significant bacteriuria (presence of $\geq 10^5$ cfu/ml of uropathogens) or low-level bacteriuria (containing between 10^3 and 10^4 cfu/ml of uropathogens). The 16S rDNA-based assay could identify the most prevalent uropathogens using probes for *Escherichia coli*, *Pseudomonas* spp., *Pseudomonas aeruginosa*, *Staphylococcus* spp., *Staphylococcus aureus*, *Enterococcus* spp. and *Streptococcus* spp. In total, 225 urinary specimens were analysed and results were compared with conventional urine culture. Using a PCR cycle threshold (Ct) value of 25 as semi-quantitative breakpoint for significant bacteriuria resulted in a sensitivity and specificity of 97% and 75%, respectively. In 78% of the samples with monomicrobial infections the assay contained probes to detect the bacteria present in the urine specimens and 98% of these uropathogens was correctly identified. Concluding, the novel presented approach can distinguish low-level and significant bacteriuria as well as detect the involved uropathogen within four hours after sampling, allowing adequate therapy decisions within the same day as well as drastically reduce consequent urine culturing.

1. Introduction

Molecular techniques are becoming an integral part of a diagnostic microbiological laboratory. Many microbiological laboratories performing real-time PCR already offer a broad panel of bacterial and viral targets. Though, for some infections conventional testing procedures are still being used. Urinary tract infections (UTIs) comprise one of the largest classes of infections occurring both in hospital and in community[1-3]. The diagnosis of UTIs is based on semi-quantitative urine culture, used as reference standard, which provides both quantification as well as identification of the uropathogen. Quantification of uropathogens is essential as different bacterial loads ($>10^3$ or 10^5 cfu/ml) are used in combination with clinical symptoms to identify UTIs in different population groups[4]. Obtaining results from a semi-quantitative culture requires at least 18 to 24 hours. In addition, previous studies have shown that depending on the population, up to 80% of urine cultures are negative, underlining the need for new, less time-consuming and labour-intensive methods[3, 5-7].

To increase the rapidity of identification of UTIs, rapid urinalysis tools are available and include for example testing for nitrite and leukocytes, and microscopic sediment analysis for bacteria and white blood cells. These screening tools are fast, but often lack sensitivity[8, 9]. Not all bacteria are capable of converting nitrate into nitrite, resulting in a false-negative test result. Particularly, *Pseudomonas aeruginosa* and Gram-positive uropathogens such as *Staphylococcus saprophyticus* and enterococci do not present this bacterial metabolism[10, 11]. Reports associated the nitrite test with a sensitivity and specificity of 45-60 % and 85-98%, respectively, leukocyte-esterase testing has been shown to have sensitivity and specificity rates of 48-86% and 17-93%, respectively[8]. The rapidity and ease of use of urine dipsticks are particularly preferred by general practitioners, whereas microbiological laboratories demand solutions designed for high-throughput analysis. In addition, within hospital settings with generally more complicated UTI's in different risk groups, a disadvantage of the dipstick testing is that the test cannot be adjusted to detect different bacterial loads (i.e.,$>10^3$ or 10^5 cfu/ml). An example of an automated urinalysis instrument is the Sysmex urine fluorescence flow cytometer (Sysmex UF-1000i). This instrument uses an algorithm which combines the quantitative detection of bacteria and white blood cells to determine if infection is present. Screening-positive samples can be further analyzed with urine culture reducing the amount of microbiology tests and minimizing unnecessary antibiotic therapy in case of screening-negative samples. Although associated with distinct advantages such as the ability of the Sysmex system to adjust settings to cover either bacterial loads $>10^3$ or 10^5 cfu/ml, this system, as well as the dipstick testing, show limitations in clinical sensitivity and do not provide

an identification of the uropathogen involved. Especially in a hospital setting, where often a larger variation in the different bacteria causing UTIs is found as compared to at the general practitioners, uropathogen identification might prove essential to aid in rapid and adequate treatment.
The aim of this study was to develop a new molecular approach accounting for both rapid semi-quantification of the bacterial load in urine as well as the identification of the uropathogen. This UTI screening assay was based on the earlier described multi-probe 16S rDNA-based real-time PCR assay using species- and genus-specific probes[12]. The most promising new feature evaluated in the current study was the semi-quantitative breakpoint to distinguish between positive and negative urinary samples. The discriminatory breakpoint was based on the scattering in cycle threshold (Ct) values of the universal 16S rDNA probe. In this study, we defined significant bacteriuria as an uropathogen load $\geq 10^5$ cfu/ml and low-level bacteriuria as a load between 10^3-10^4 cfu/ml. Bacterial loads below 10^3 cfu/ml were considered to be no UTI. The other purpose of the UTI screening tool was to offer a rapid indication about the causative agent. The pathogens selected as target in the real-time PCR assay were *Escherichia coli*, *Pseudomonas* spp., *P. aeruginosa*, *Staphylococcus* spp., *Staphylococcus aureus*, *Enterococcus* spp., and *Streptococcus* spp.

2. Materials and methods

Clinical samples

We included 225 clinical urine samples submitted to the Medical Microbiology Laboratory of the Maastricht University Medical Center (MUMC+, Maastricht, The Netherlands). The samples were collected from in- and outpatients, which had a clinical suspicion of a UTI. Conventional processing of the urine specimens consisted of dipstick testing for nitrite and/or further pathogen identification by Gram stain and biochemical testing. Samples were cultured using standard microbiological methods. Identification and antibiotic susceptibility testing was performed with the Phoenix system (BD Diagnostic Systems). Colony counts of $\geq 10^5$ cfu/ml were considered as the presence of significant bacteriuria. All samples were used according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies.

Processing of urine samples

1 ml of urine sample was centrifuged for 5 min (13,400 x g). The supernatant was removed and the remaining pellet was washed with 900 µl phosphate buffered saline (PBS) and centrifuged for 5 min (13,400 x g). Again, the

supernatant was removed and the pellet was incubated in a lysozyme-lysostaphyn (0.1 mg - 0.01 mg) mixture for 15 min at 37°C. After this pre-treatment, DNA isolation was performed with the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Finally, DNA was eluted in 100 µl of nuclease-free H_2O. To check for optimal DNA isolation and inhibition during amplification each sample was spiked with mCMVgB.

Multiprobe assay

The primers and the universal bacterial TaqMan probe have been described previously[13]. The probes for *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp., *Streptococcus* spp. were previously designed and described[12]. Each test contained 5 µl purified sample and 20 µl reaction mixture. The reaction mixture contained 12.5 µl of Taqman Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA), 0.9 µM of forward primer, 0.6 µM of reverse primer, and 0.2 µM of each probe. There were four separate reactions: I) universal probe, *Pseudomonas* spp. probe and *E. coli* probe, II) *P. aeruginosa* probe, III) *Staphylococcus* spp. probe, the *S. aureus* probe and the *Enterococcus* spp. probe, IV) *Streptococcus* spp. probe. Reactions were performed on the ABI PRISM® 7900 real-time PCR System (Applied Biosystems, Foster City, CA, USA) and optimal thermal cycling conditions were as follows: 10 min at 50°C, initial denaturation at 95°C for 15 min, 42 cycles of denaturation for 15 s at 95°C and annealing at 60°C for 1 min. Cycle threshold (Ct), the cycle number at which amplicon fluorescence exceeded the preset detection threshold, was recorded for all samples. The threshold for the Ct analysis was manually adjusted to 0.1, together with the baseline start and end (cycle): 6-15.

Statistical analysis

The positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity were calculated for the real-time PCR using the presence of ≥ 10^5 cfu/ml in culture as gold standard. For the statistical analysis, data were analyzed using SPSS software, version PASW Statistics 18 (SPSS Inc, Chicago IL, USA). Receiver Operating Characteristics (ROC) curve analysis was performed to determine a real-time PCR-based semi-quantitative breakpoint. The Mann-Whitney U Test and Kruskal-Wallis Test were used to enable correlation of the PCR Ct values with the bacterial load determined in culture. A p-value of < 0.05 was considered significant.

3. Results

Population and assay characteristics

The collection of urine specimens consisted of 225 samples, of which 186 samples were analyzed retrospectively using the multiprobe assay. Six samples were excluded because of insufficient sample volume, 32 urine specimens were determined as skin flora and one sample was excluded because of a fungal infection. In our test collection, the prevalence of a positive urine culture was 39%. The complete assay including DNA extraction and real-time PCR could be performed within four hours.

A semi-quantitative breakpoint for the detection of significant bacteriuria

Within the Maastricht University Medical Center, urine samples (in conjunction with the matching clinical symptoms) are categorized into two groups: UTI if bacteria are present at a concentration equal or higher than 10^5 cfu/ml and no UTI if the concentration of the bacteria is less than 10^5 cfu/ml. In this study, we also wanted to determine a semi-quantitative real-time PCR-based cut-off value to distinguish between UTI and no UTI. Therefore, the universal 16S rDNA probe was used as target, and Ct values were related to the cut-off value applied in culture. To evaluate the accuracy and the discriminating power of our diagnostic test, a ROC curve was made (Figure 1). The real-time PCR assay showed a good accuracy (AUC=0.93).
Following, the threshold used to discriminate between positive and negative urine samples was set to a Ct value of 25, resulting in a significant difference in Ct value between the UTI and no UTI group (Figure 2). Using this cut-off value, a sensitivity and specificity of 97% and 75% respectively, could be reached. The positive and negative predictive values were 71% and 98%, respectively (Table 1).

Table 1. Performance characteristics of the diagnostic test (real-time PCR assay, universal probe) using Ct 25 as semi-quantitative cut-off value.

Diagnostic test result	Urine culture		Sens	Spec	PPV	NPV
	≥10^5 cfu/ml	<10^5 cfu/ml				
PCR positive	70	29	97	75	71	98
PCR negative	2	85				

PPV = positive predictive value; NPV = negative predictive value; Sens = sensitivity; Spec = specificity

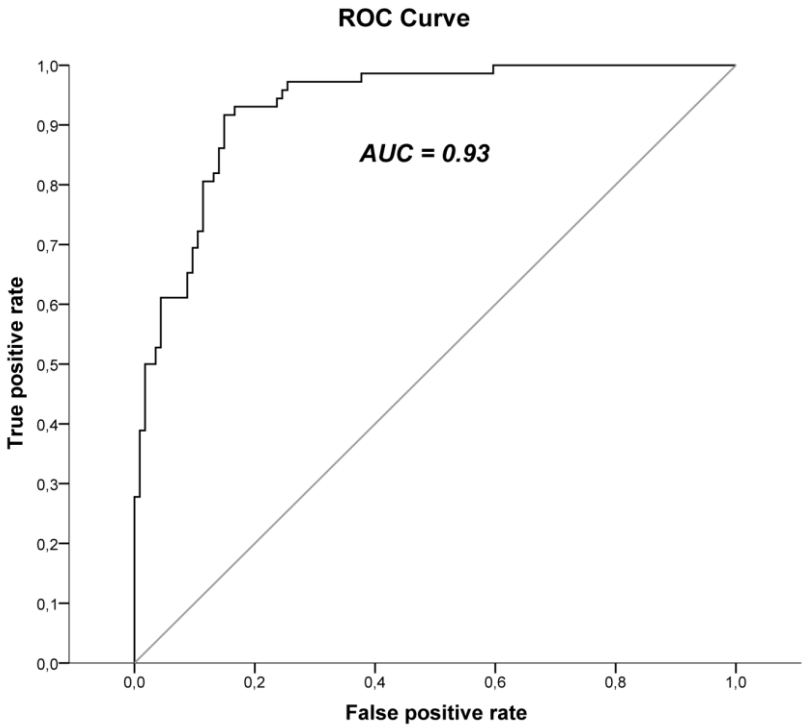

Figure 1. Receiver Operating Characteristic (ROC) decision plot obtained by using the real-time PCR Ct values (universal probe) versus urine culture results (Cut-off value of $\geq 10^5$ cfu/ml).

As shown in Figure 2, two culture-positive samples were false-negative in our diagnostic PCR assay (Ct value of 29.56 and 26.62). An internal amplification control showed that the late signals were not due to inhibition (data not shown). The first sample contained *Enterococcus faecalis* (10^5 cfu/ml), and the second *Enterobacter cloacae* (>10^5 cfu/ml). Furthermore, we observed 29 false-positive results with PCR, of which in five cases the culture report mentioned the presence of a specific pathogen between 10^4 and 10^5 cfu/ml. In the remaining 24 cases no bacteria were found in culture. Adjusting the cut-off value to Ct 30 could resolve the two false-negative results. However, then the amount of false-positive isolates would increase to 72 cases. In Table 2, a summary is given of the performance characteristics of the PCR assay using alternative cut-off values.

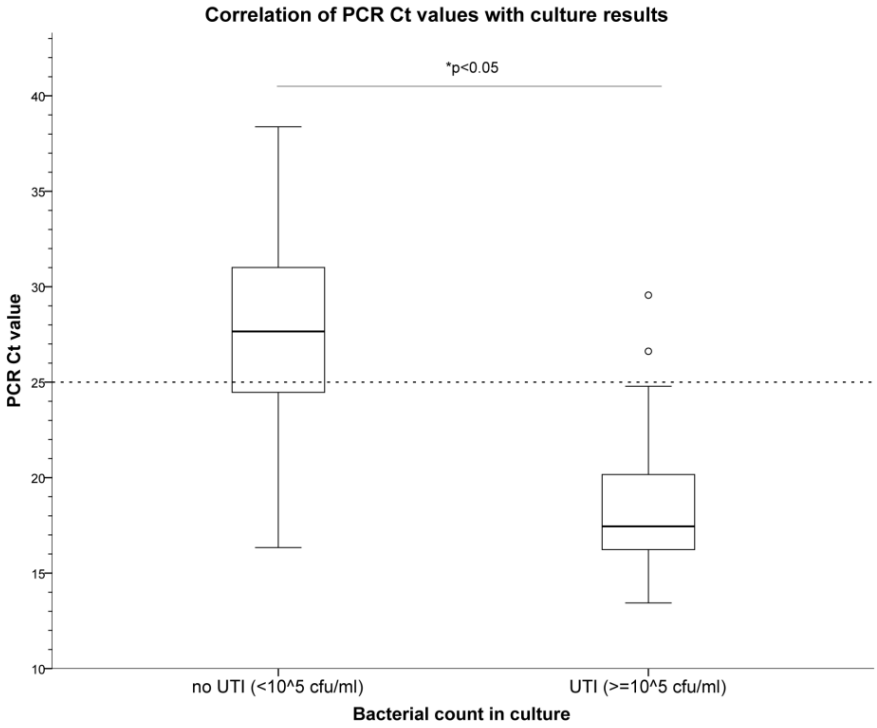

Figure 2. Correlation of Ct values with culture results. Based on culture, samples were categorized into two groups: UTI (≥10^5 cfu/ml) and no UTI (<10^5 cfu/ml). The universal 16S rDNA probe, of which a Ct value of 25 was set as breakpoint, was used to distinguish between positive and negative samples (line in dots). * p<0.05 (non-parametric Mann-Whitney Test)

An alternative cut-off value for the diagnosis of UTI

In different guidelines as well as in different population groups, cut-off values of 10^3-10^4 cfu/ml may be considered for the definition of UTI. In Figure 3, the Ct values of the universal probe were scattered against the bacterial load determined in culture. Both the presence of ≥10^5 cfu/ml of a single uropathogen as well as 10^3 and 10^4 cfu/ml were studied. Statistical analysis showed that there was a significant (p<0.05) difference in Ct values between the three categories. Based on the Ct value of the universal 16S rDNA probe, a differentiation can be made between the different cut-off bacterial loads.

Table 2. Comparison of performance characteristics of the diagnostic test (real-time PCR assay, universal probe) using different Ct cut-off values (25-30). Ct 25 was used as definitive cut-off value (marked in grey).

Cut-off value	Sensitivity	Specificity	PPV	NPV
24	94	76	72	96
25	97	75	71	98
26	97	68	65	97
27	99	56	59	98
28	99	48	55	98
29	99	42	52	98
30	100	37	50	100

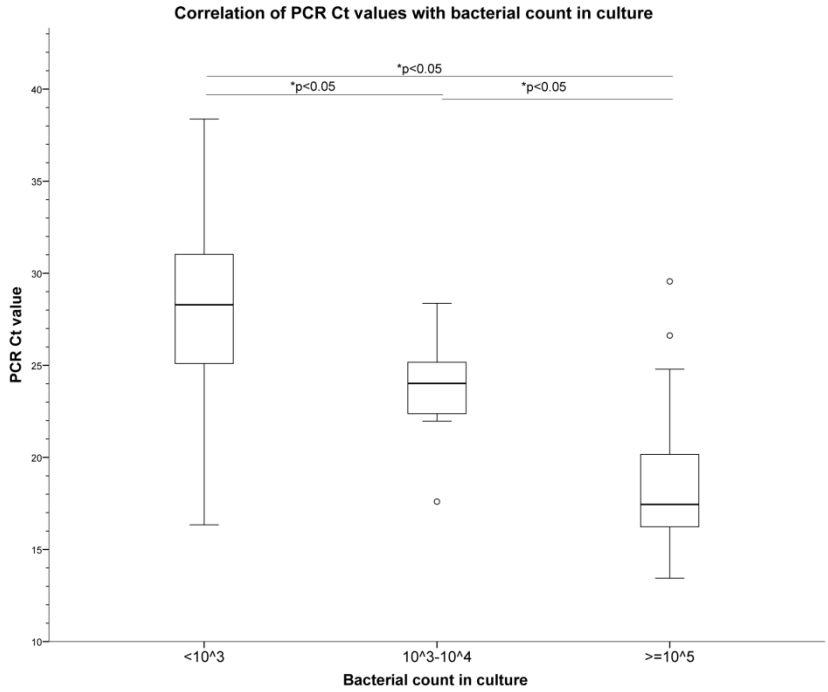

Figure 3. Boxplot showing the correlation of PCR Ct values with bacterial load determined in culture. Culture results were grouped into three categories: $<10^3$ cfu/ml, 10^3-10^4 cfu/ml and $\geq 10^5$ cfu/ml. Statistical significance was determined performing Kruskal-Wallis Test.

Molecular probes for the identification of the most prevalent uropathogens

The real-time PCR assay was, in addition to the universal 16S rDNA probe, complemented with seven genus- or species-specific probes. In this way, parallel to the differentiation between UTI and no UTI with the eubacterial 16S probe, the multiprobe assay also offered a first identification of clinically relevant uropathogens. We analyzed 186 urine specimens, of which 64 samples were considered as significant bacteriuria because of the presence of $\geq 10^5$ cfu/ml of a single uropathogen (Table 3. I). The Ct value derived from the universal 16S rDNA probe was used as reference point. If no other probe generated a signal within four Ct proximity of the universal probe, the pathogen was identified as 'other', being a pathogen not included in the bacterial panel. The panel of probes could identify 76.5% of the pathogens present in the samples with monomicrobial infections. *E. coli* was the most frequently found uropathogen (59%), and was 100% correctly identified with the specific probe. The other probes targeting *Staphylococcus* spp., *Enterococcus* spp., *Pseudomonas* spp., *P. aeruginosa* also showed a detection rate of 100%. One sample containing a *Streptococcus* spp. was missed, resulting in a detection rate of 75%. In eight isolates, a polymicrobial infection was shown (Table 3. II). The assay was able to identify more than one microorganism within the same sample. As shown in Table 3 (III), in seven cases pathogens were found both in culture and in PCR, but were, because of the presence of low counts, considered as no UTI. In summary, the assay as identification tool achieved an agreement of 98% in the samples with monomicrobial infections.

Table 3. Pathogen identification of the 72 growth-positive urine specimens (I. Monomicrobial infections, II. Polymicrobial infections, III. Pathogens present <10^5 cfu/ml, no UTI))

CULTURE	PCR		
Pathogen	ID probe	n	Concordance (%)
I. Monomicrobial UTI (n=64)			
Acinetobacter baumanii	Other[a]	1	
beta-hemolytic *Streptococcus* spp.	*Streptococcus* spp.	3/4	75%
Citrobacter braakii	Other	1	
Citrobacter freundii	Other	1	
Coagulase-negative *Staphylococcus* spp.	*Staphylococcus* spp.	1/1	100%
Enterobacter cloacae	Other	2	
Enterococcus faecalis	*Enterococcus* spp.	6/6	100%
Escherichia coli	*Escherichia coli*	38/38	100%
Klebsiella pneumoniae	Other	7	
Proteus mirabilis	Other	2	
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*[b]	1/1	100%
Total (pathogens included in PCR panel)		49/50	98%
II. Polymicrobial UTI (n=8)			
Citrobacter freundii, Enterococcus faecalis	Other, *Enterococcus* spp., *Staphylococcus* spp.		
Escherichia coli, Enterococcus faecalis, Candida spp.	*Escherichia coli, Enterococcus* spp.		
Escherichia coli, Pseudomonas aeruginosa, Enterococcus faecalis	*Escherichia coli, Enterococcus* spp., *Pseudomonas aeruginosa*		
Escherichia coli, skin flora	Other, *Staphylococcus* spp.		
Klebsiella oxytoca, Escherichia coli	*Escherichia coli, Streptococcus* spp.		
Klebsiella pneumoniae, Proteus mirabilis	Other		
Klebsiella pneumoniae, Proteus mirabilis	Other, *Enterococcus* spp.		
Proteus vulgaris, Enterobacter aerogenes	Other		
III. Uropathogens present <10^5 cfu/ml			
Escherichia coli	*Escherichia coli*	3/3	
Klebsiella pneumoniae	Other	1	
Proteus mirabilis	Other	2	
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	2/2	

[a] None of the species- or genus-specific probes generated a signal, only the universal 16S rDNA probe was positive, indicating the presence of another pathogen, not included in the bacterial panel of the real-time PCR assay. [b] The isolate was identified as *P. aeruginosa*, and this was shown by positive signals from both the *Pseudomonas* spp. and the *P. aeruginosa* probe.

4. Discussion

In this study, we present a novel molecular approach which utilised all capabilities of broad-spectrum real-time PCR to aid in the diagnosis of UTIs. The assay detecting the majority of uropathogens as well as a universal probe detecting eubacteria was used to establish a breakpoint that could distinguish urinary samples with significant, or low or no bacteriuria. Secondly, this screening assay was reinforced with rapid uropathogen identification. The assay presented in this study is based on the detection of 16S rDNA gene signatures in real-time PCR. The universal probe targeting the 16S rDNA gene was used to establish a discriminatory set point between bacteriuria and no bacteriuria. In order to prevent positive samples to be falsely considered as negative, the assay needed to have a high sensitivity and negative predictive value. A Ct value of 30 could be applied to achieve a sensitivity and NPV of 100%. However, we selected a Ct value of 25 as breakpoint because we wanted to find a proper balance between the amount of false-negative and false-positive samples. Ultimately, screening of urinary samples with our assay resulted in a sensitivity and NPV of 97% and 98%, respectively.

Thus far, another rapid approach that has been presented to distinguish bacteriuria from no bacteriuria has been the Sysmex UF-100/500/1000i urine flow cytometer. Many different studies have shown that performance characteristics could be quite different because of variable cut-off values for white blood cells (WBC) and bacteria[3, 6, 14-17]. Traditionally, often significant bacteriuria is defined as the presence of $\geq 10^5$ cfu/ml[18, 19]. In recent years, both the American Society of Microbiology (ASM) and European urinalysis guidelines have recommended colony counts of $\geq 10^3$ cfu/ml of uropathogens to be reported as UTI[20]. Therefore, we investigated if further subdivision was possible with this UTI-screening assay. Indeed, we could find a significant difference in Ct value when three instead of two categories were made, as shown in Figure 3. But, lowering the cut-off value in culture, would result in an increase in the amount of contaminants that would be reported.

In order to establish whether the bacterial load in urine is a result of a high load of uropathogens or of contaminating skin flora, the second part of the assay, i.e. the identification of the pathogen, can be of great value. We saw that the absolute Ct value of the universal probe can be linked to the absolute Ct value of the specific probes. So, if the urinary sample contained a microorganism not included in the panel, the Ct value of the universal probe is that low, indicating the presence of another pathogen. From our results it became apparent that when the specific probe generated a signal within four Ct of the universal probe, we could define this as the causative agent. The current panel included probes for the most frequently found uropathogens i.e. *E. coli*, *Pseudomonas* spp., and *Staphylococcus* spp. A possible future target could be *Klebsiella pneumonia*

since the prevalence was similar to *Enterococcus* spp. As shown in Table 3 III, the assay could also identify polymicrobial infections. In these cases, it was also more likely to find contaminants such as *Staphylococcus* spp. and *Streptococcus* spp. A report by Wu *et al.* presented a similar approach, using FISH with specific probes for the identification of uropathogens (*E. coli*, *E. faecalis* and *S. aureus*). The sensitivity of the *E. coli* specific probe was 95%, whereas the specificity of the *S. aureus* probe was 98%[21]. The clinical relevance of the *S. aureus* probe can be discussed since it was not detected as causative agent in our sample collection. Thus in future studies, this probe could potentially be deleted from the current assay. Lehmann *et al.* developed a real-time PCR consisting of species-specific probes for 15 pathogens and achieved a sensitivity and specificity of 90% and 87%, respectively [22]. Taken together, our novel approach can be of significant relevance for the rapid diagnosis of UTI because of the ability to distinguish between lower counts and significant bacteriuria, and this combined with the identification of the uropathogen. Identification of a uropathogen is especially vital in hospital settings because of a more diverse UTI etiology.

Overall, the UTI screening and identification assay showed good performance characteristics. In 76.5% of the monomicrobial infections, the bacterial panel correctly diagnosed the causative agent and results were available as soon as within four hours. One isolate could not be identified as *Streptococcus* spp. The remaining 22% contained microorganisms not included in the bacterial panel. The limited number of bacterial targets could be a limitation of the assay, though the most important uropathogens are included. Another potential limitation of the assay is that the use of real-time PCR supplemented with molecular probes is more expensive than urine culture. Though, future applications concerning miniaturisation of PCR such as micro fluidic digital PCR could be used to offer rapidity, high-throughput and lower running costs, making this assay more cost-effective. The most important feature of the assay was the capability to categorize into no bacteriuria ($<10^3$ cfu/ml), low-level bacteriuria (10^3-10^4 cfu/ml) and significant bacteriuria ($\geq 10^5$ cfu/ml). Additionally, in this study, screening of urinary specimens would have reduced the amount of urine specimens to be cultured with 47%. In summary, the assay could be of great value to improve the diagnosis and therapy of UTIs, especially in hospital settings.

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Chapter 5

Rapid identification and genotypic antibiotic resistance determination of staphylococci

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Abstract

A genotypic approach for positive blood cultures was developed for the rapid identification and determination of the most relevant antimicrobial resistance phenotypes occurring in staphylococci. The identification of the staphylococci was based on 16S rDNA gene amplification and was achieved using probes for *Staphylococcus* species and *Staphylococcus aureus*. Primers targeting the genes *mecA*, *bla*$_Z$, *aac(6')-aph(2")*, *vanA*, *ermA*, *ermC* and *msrA* were selected to enable profiling of the staphylococcal antimicrobial resistance profile. Current routinely used phenotypic methods require further culturing steps resulting in a delay of at least 24 hours. In this study, the identification panel achieved a correlation of 100% with conventional bacterial identification. Next, we found a categorical agreement of 96% between the conventional antibiotic susceptibility testing and the genotypic resistance profiles detected with our real-time PCR assay. Combined, these data showed a high agreement between conventional phenotypic and genotypic testing and could drastically reduce the time needed for analysis. The use of both this identification and antibiotic resistance determination panel allowed the analysis of positive blood cultures within two hours and could therefore be of significant relevance for improvement of diagnostic methods involved in bloodstream infections.

1. Introduction

In patients with a suspicion of bloodstream infection (BSI) information regarding the identification of the causative agent and the microorganisms' antimicrobial resistance profile is required. The importance of obtaining an antimicrobial resistance profile has been demonstrated by reports showing that the administration of appropriate antibiotics is correlated with a decrease in mortality[1, 2]. Conventional antimicrobial susceptibility testing (AST) methods such as the agar dilution test, microdilution assay, E-test and disk diffusion test, still involve pure subculturing and therefore take up to 24 hours before initial results are known. So, infections are treated empirically until final results are available. Based on the nature of the test, the use of a rapid, DNA-based test could shorten the time between empirical and streamlined antimicrobial therapy, and would thus be beneficial for the outcome of the patient. Genetic methods for the detection of antimicrobial resistance still need a careful exploration, though some resistance genes (*mecA*, *vanA*, *vanB*)[3] are firmly associated with phenotypic resistance[4]. In fact, because of difficulties in phenotypic susceptibility testing for methicillin resistance, the detection of the *mecA* gene is now recognized as the most definitive method[5]. Despite the promise of this direct approach, potential limitations such as the large amount of resistance mechanisms that would need to be detected or the possibility that some resistance genes may not be expressed or may not result in phenotypic resistance, have to be overcome[5].

The most important Gram-positive bacteria causing bloodstream infections are the staphylococci, especially *Staphylococcus aureus* and *Staphylococcus epidermidis*, which are associated with the highest pathogenicity[6]. Beta-lactam antibiotics, aminoglycosides, glycopeptides and macrolide, lincosamide, and streptogramin (MLS) antibiotics are all commonly used to treat infections caused by staphylococci. Both *S. aureus* and coagulase-negative staphylococci (CoNS) are known to accumulate more than one antibiotic resistance determinant, resulting in multi-drug resistant strains. Within the group of the beta-lactam antibiotics, resistance to oxacillin (methicillin) and penicillin is most frequently observed. Oxacillin resistance in staphylococci is conferred by the chromosomally located *mecA* gene, which encodes for an altered penicillin-binding protein PBP2a[7-9]. Hence, the resistance mechanism responsible for the lowered affinity for beta-lactam antibiotics is based on the occurrence of only one resistance gene determinant. But, resistance mechanisms are mostly established by the presence of more than one antibiotic resistance gene. Examples are multiple-gene-based resistance mechanisms for vancomycin[10, 11] and MLS antibiotics. Within the group of the MLS antibiotics different mechanisms of acquired resistance have been found in Gram-positive bacteria[11]. Target modification by methylases, which are encoded by

erythromycin ribosome methylation (erm) genes, is the most common mechanism found. Another relevant possibility is the presence of efflux proteins, encoded by for instance the *msrA* gene. Presence of the *msrA* gene confers resistance to both macrolides and streptogramin B antibiotics (MS phenotype)[11, 12].

The aim of this study was to offer a clinically relevant diagnostic approach for bacterial infections using real-time PCR directly on positive blood cultures for on the one hand the identification of staphylococci and on the other hand the detection of the most important antibiotic resistance genes and to compare these results with conventional AST for beta-lactam antibiotics, glycopeptides, aminoglycosides and MLS antibiotics. The antibiotic resistance genes tested were selected based on their clinical relevance and included *mecA*, *bla*$_Z$, *aac(6')-aph(2'')*,*vanA*, *ermA*, *ermC* and *msrA*. The use of this novel antibiotic resistance testing assay in combination with rapid identification could drastically improve the diagnosis and therapy of bloodstream infections.

2. Materials and Methods

Clinical samples and sample preparation

Between September 2008 and March 2009, a total of 114 *Staphylococcus* spp. isolates from positive blood cultures were collected at the department of Medical Microbiology, Maastricht University Medical Center (MUMC+, Maastricht, The Netherlands), and included in this study. For the identification, all samples were analyzed with standard conventional testing including culture, Gram-staining and biochemical tests (catalase, coagulase and DNase production). A limited sample preparation step was performed prior to analysis of the staphylococcal isolates with the PCR-based identification assay, and our new antimicrobial resistance testing method. Briefly, an aliquot (0.1 ml) of the blood cultures was 1:100 diluted in sterile 0.9% NaCl. After a centrifugation step of 5 min (13,400 x g), the bacterial pellet was resuspended in 100 µl of nuclease-free water. All samples were used according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies.

Bacterial identification with multi-probe assay

As previously described in Hansen *et al.*, we developed a real-time PCR supplemented with molecular probes for the identification of different bacterial pathogens. For this study, one universal 16S rDNA probe and two specific probes targeting *Staphylococcus* spp. and *S. aureus* were used[13]. In addition,

the primers and probe for *femA* were included in the assay for the identification of *S. aureus* [14].

Conventional antimicrobial susceptibility testing

AST was performed using routine phenotypic methods according to the CLSI guidelines. A standard inoculum in Phoenix ID Broth (BD Diagnostic Systems) was prepared from the bacteria grown on the agar medium and inoculated into Phoenix panels, following the manufacturer's recommendations. In case of discrepancies between the phenotypic and genotypic method, microbroth dilution was used, as described in the CLSI guidelines[15].

New antimicrobial resistance testing real-time PCR assay

We selected five antibiotic resistance phenotypes as target, including oxacillin, penicillin, gentamicin, vancomycin and erythromycin, and these were represented by seven antibiotic resistance genes (i.e., *mecA*, *bla$_Z$*, *aac(6')-aph(2")*, *vanA*, *ermA*, *ermC* and *msrA*, respectively). The real-time PCR assay was based on non-specific fluorescent dye-based chemistries using the EvaGreen® dye. The primers for the antibiotic resistance genes *bla$_Z$*, *aac(6')-aph(2")*, *mecA*, *ermA*, *ermC*, *msrA* and *vanA* were described previously (Table 1). Each reaction contained a 5 µl purified sample and a 20 µl reaction mixture. The reaction mixture contained 5 µl HOT FIREPol® EvaGreen qPCR Mix Plus (Rox) (Solis BioDyne, Tartu, Estonia) and 0.6 µM forward and reverse primer. The real-time PCR was performed using the Bio-Rad IQ5 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The following optimal thermal cycling conditions were used: cycle 1 (95°C for 30 s), cycle 2 (95°C for 5 min), cycle 3 42 x (step 1: 95°C for 15 s – step 2: 60°C for 1 min), data collection, cycle 4 (95°C for 1 min), cycle 5 (50°C for 1 min), cycle 6 60 x (65°C-94.5°C for 10 s) with increasing the set point temperature after cycle 2 by 0.5 °C, melt curve data collection. The cycle threshold (Ct) value and melting peak (Tm ± 0.5°C) specific for each target was recorded for all samples (Tm *bla$_Z$* :80°C, Tm *aac(6')-aph(2")*: 78°C , Tm *mecA*: 77°C, Tm *ermA*: 81.5°C, Tm *ermC*: 78.5°C, Tm *msrA*: 83°C).

Data analysis

The results of our new genotypic method for antimicrobial resistance testing (susceptible or resistant) were compared with the results of conventional AST (susceptible, intermediate or resistant) for categorical agreement. Performance characteristics of our new test were determined and included sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

Additionally, major errors (false-resistant result) and very majors errors (false-susceptible result) were calculated as discussed in Jorgensen *et al* [16]. Minor errors (false result concerning an intermediate result) were not defined since real-time PCR could only be used to screen for the presence or absence of a specific resistance gene.

Table 1. Staphylococcal antibiotic resistance gene-specific primers used in this study.

Oligonucleotide	Resistance phenotype[a]	Sequence (5'-3')	Reference
mecA_f	OXA	TGAAGTGGTAAAATGGTAATATCGACTTAA	[14]
mecA_r		TATTTCGACTGCTACTCTAGCAAAGAA	
bla$_Z$_f	PEN	ACTTCAACACCTGCTGCTTTC	[6]
bla$_Z$_r		TGACCACTTTTATCAGCAACC	
aac(6')-aph(2'')_f	GEN	TTGGGAAGATGAAGTTTTTAGA	[6]
aac(6')-aph(2'')_r		CCTTTACTCCAATAATTTGGCT	
vanA_f	VAN	CATGAATAGAATAAAAGTTGCAATA	
vanA_r		CCCCTTTAACGCTAATACGATCAA	
ermA_f	ERY	TATCTTATCGTTGAGAAGGGATT	[6]
ermA_r		CTACACTTGGCTTAGGATGAAA	
ermC_f	ERY	CTTGTTGATCACGATAATTTCC	[6]
ermC_r		ATCTTTTAGCAAACCCGTATTC	
msrA_f	ERY	TCCAATCATTGCACAAAATC	[6]
msrA_r		AATTCCCTCTATTTGGTGGT	

[a] OXA, oxacillin; PEN, penicillin; GEN, gentamicin; VAN, vancomycin; ERY, erythromycin;

3. Results

A total of 114 staphylococcal isolates were included in this study, of which 33 isolates were identified as *S. aureus* and the remaining 81 isolates as CoNS using conventional culturing and identification techniques. Using our multi-probe identification assay, in which *S. aureus* and other *Staphylococcus* spp. could be distinguished, an agreement of 100% could be reached and this within two hours. The correlation between the AST and our real-time PCR assay is presented in Table 2. Each strain was tested for each target, and this resulted in a categorical agreement of 96%. No strains were positive for the *vanA* resistance gene, the specificity of the primers was confirmed with a positive control (data not shown). In total, we defined 21 discrepancies as major error and one discrepancy as very major error.

The correlation between the phenotypic and genotypic approach, in which there was only one potential causative antibiotic resistance gene, did not show any false-susceptible strains. Among the 65 oxacillin-susceptible staphylococcal strains three carriers of the *mecA* gene were found (repeated MIC values of

≤0.25 mg/L, 0.25 mg/L and 0.12 mg/L). Screening for gentamicin resistance resulted in the misidentification of five out of 82 susceptible strains (repeated MIC values of <0.06 mg/L, <0.06 mg/L, 1, ≤0.25 mg/L and ≤0.25 mg/L). Among the 27 penicillin-susceptible strains in 11 cases the *bla*$_Z$ gene was detected (repeated MIC values between 0.015 mg/L and 0.12 mg/L).

Table 2. Correlation between AST and the genotypic antibiotic resistance testing approach using real-time PCR.

	Genotypic antibiotic resistance testing							
	mecA		*blaZ*		*aac(6')-aph(2'')*		*ermA/ermC/msrA*	
	oxacillin		penicillin		gentamicin		erythromcyin	
AST[a]	PCR+	PCR-	PCR+	PCR-	PCR+	PCR-	PCR+	PCR-
resistant	49	0	87	0	32	0	50	1
susceptible	3	62	11	16	5	77	2	54
Sens (%)	100		100		100		98	
Spec (%)	95		59		94		96	
PPV (%)	84		89		86		96	
NPV (%)	100		100		100		98	

[a], the category Intermediate (I) was excluded for the analysis of this antibiotic phenotype since this genotype is not distinguishable with real-time PCR (n=7)
PPV = positive predictive value; NPV = negative predictive value; Sens = sensitivity; Spec = specificity

Table 3. Distribution of the different antibiotic resistance genes for the erythromycin resistance phenotype.

	Erythromycin				
	Genotypic antibiotic resistance testing				
	ermA	*ermC*	*msrA*	*ermA+ermC*	*ermC+msrA*
AST: resistant	7	23	13	2	5

To screen for resistance to erythromycin, we selected three antibiotic resistance genes including *ermA*, *ermC* and *msrA*. A total of 51 strains were erythromycin-resistant. The prevalence of the three different antibiotic resistance genes in our study collection is shown in Table 3. In seven of these strains *ermA* was present, in 23 strains *ermC* was present and thirteen strains contained the *msrA* gene. Additionally, two isolates carried both *ermA* and *ermC*, and another five isolates carried both *ermC* and *msrA*. One resistant strain, with a MIC value of >32 mg/L, was missed using the genotypic real-time PCR. In two susceptible isolates *ermA* and *ermC*, respectively were detected. A repeated MIC determination confirmed the AST (MIC ≤0.25 mg/L). Thus, the assay for erythromycin resistance testing correlated in 97% with the antibiotic susceptibility testing.

4. Discussion

This study described a novel diagnostic approach for the determination of both the identification as well as the antimicrobial resistance profile of staphylococcal isolates from positive blood cultures. The identification assay could distinguish *S. aureus* from other *Staphylococcus* spp., and included a universal 16S rDNA probe enabling detection of other bacteria as well. Among these, three probes (universal 16S rDNA, *Staphylococcus* spp. and *S. aureus* probe) were originally included in the more extended bacterial panel of the multi-probe assay that was developed for the identification of the most clinically relevant bacteria causing bloodstream infections[13]. The identification assay was combined with a real-time PCR assay detecting seven antibiotic resistance genes. The panel of antibiotics was selected based on clinical importance and was not intended to provide a complete coverage of all AST results. In our study collection, a 96% categorical agreement between phenotypic and genotypic testing was achieved. The main weakness of conventional AST is the time-consuming processing of patient specimens in order to obtain pure cultures. Previous reports that focused on antibiotic resistance determination in staphylococci were mainly performed on bacterial suspensions after subculturing[17-19]. Paule *et al.* developed a real-time PCR for positive blood cultures, but focused on the detection of the *mecA* gene for oxacillin resistance[20]. The group of Martineau *et al.* reported multiplex PCR assays including a more enlarged panel of antibiotic resistance genes, of which most of the primers in our study were selected[6, 21]. To drastically improve the turnaround time, we combined staphylococcal identification with determination of the antimicrobial resistance profile, and tested this directly from positive blood cultures. In this way, after approximately two hours, a distinction between *S. aureus* and other *Staphylococcus* spp. could be made, together with the presence or absence of seven antibiotic resistance genes. We selected three genes to compare genotypic resistance, encoded by

ermA, *ermC* and *msrA,* with the erythromycin-resistant phenotype. Overall, except for one isolate, the selection of the genes *ermA*, *ermC* and *msrA* seemed to be sufficient for an accurate determination of erythromycin resistance in staphylococci. The presence of *ermC* (45%) and *msrA* (35%) was highest in our collection of staphylococci, and these were only found in CoNS. Other reports by Westh *et al.* and Martineau *et al.* also found high incidences of *ermC* in erythromycin-resistant CoNS[6, 22]. Eady *et al.* and Martineau *et al.* also included the detection of *ermB*, but since not one resistant strain carrying this gene could be found, we did not add this gene in our study[21, 23].

An important consequence of genotypic testing that one has to keep in mind is that the presence of a certain gene not necessarily results in phenotypic resistance. An example of this potential induction of resistance was seen when testing for oxacillin resistance. Whereas phenotypic testing identified three CoNS isolates as oxacillin-susceptible, all these isolates carried the *mecA* gene, described in literature as cryptically oxacillin-resistant strains (*mecA*-positive, but non-PBP2a-producing strain)[24]. The unstable nature of oxacillin resistance was shown previously and could explain the lack of production of PBP2a and following the loss of resistance[25, 26]. Therefore, the recognition of these cryptically oxacillin-resistant strains could be of use since they could regain their ability to produce PBP2a. In this context, the genetic detection of resistance can contribute to further investigation of the underlying nature of resistance in bacterial strains.

Of course, an important aspect that has to be well considered when designing a genotypic test for diagnostic purpose in a clinical setting is costs and laboratory equipment. In the past, Perreten *et al.* described the design of a microarray capable of detecting 90 antibiotic resistance genes involved in Gram-positive bacteria[27]. Although this approach can be of major value in surveillance programs and for instance food safety, a diagnostic resistance tool for clinical specimens often does not need this high-output approach. In most cases, and depending on the pathogen involved, information about a restricted panel of antibiotics is requested for further streamlining of the initial empirical therapy. Furthermore, microarray-based technologies are associated with high costs, and are often not compatible with standard microbiology laboratory equipment.

In summary, the combination of pathogen identification and antibiotic resistance determination of staphylococci, within two hours is of significant value for the diagnosis of bacterial infections. Furthermore, the 96% agreement between our genotypic test and conventional AST meets the selection criteria for an acceptable AST system, stated by Jorgensen[16]. In future, more bacterial pathogens could be analyzed with this rapid diagnostic assay if both more identification targets as well as more antibiotic resistance genes are added. Hence, after two hours of analysis, results can be processed by the clinician, enabling more adequate therapy decisions within less time.

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Chapter 6

General discussion & summary

A wide-ranging collection of bacteria, viruses, fungi and parasites are responsible for the estimated 15 million deaths each year due to infectious diseases[1]. Apart from the high morbidity and mortality numbers, infectious diseases can cause severe disability and have also been associated with a high socio-economic burden on the society[1]. Controlling infectious diseases is complicated and is depending on different aspects, including prevention, diagnosis and treatment. The success rate of therapy is partly influenced by a rapid and accurate diagnosis of the etiologic agent and therefore, rapid methods for the diagnosis of infectious diseases are important. New diagnostic techniques should contribute to the prevention of deaths and improve quality of life drastically. Furthermore, a rapid intervention is needed to ensure the prevention of disease spread and to anticipate on the development of drug-resistant strains of certain pathogens. Although culture-based methods are commonly considered as the gold standard for the detection and identification of pathogens, the substantial delay in time to results and the insensitivity towards fastidious or slow-growing microorganisms has challenged researchers to improve current pathogen diagnostics. Molecular methods for detection and identification of bacterial and fungal microorganisms have been widely developed, and already changed the clinical practice of infectious diseases. Both protein-based (e.g., mass spectroscopy) and nucleic acid-based (amplification and/or hybridization) identification assays were introduced and are associated with increased sensitivity and specificity, detection of fastidious and slow-growing microorganisms and decreased turnaround time. But, these novel techniques are also hampered by some limitations such as the potential of false-positive results due to contamination, the impossibility to distinguish between dead and viable cells, the lack of a reference standard, and the inability to provide a full antimicrobial resistance profile. Therefore, further studies are needed to expand the current knowledge and experience related to molecular diagnostics of bacteria and fungi in infectious diseases.

From that perspective, the studies performed in this thesis focused on the improvement of detection methods for bacterial and fungal microorganisms. For this purpose, new techniques for implementation in microbiological diagnostics were searched in terms of extraction of bacterial and fungal DNA from different clinical samples, identification of pathogens using nucleic acid amplification, and determination of drug resistance using genotypic detection of antibiotic resistance genes.

The extraction of bacterial and fungal DNA from different patient specimens

Initially, a closer look was taken at sample preparation methods for the selective extraction of microbial DNA. We studied the effect of different sample preparation and DNA isolation methods for the selective extraction of bacterial DNA from whole blood samples **(Chapter 2a, 2b).** Implementation of rapid molecular testing methods directly from whole blood specimens instead of using cultured materials, could contribute to a strikingly faster diagnosis of bacterial and fungal infections permitting an earlier administration of the appropriate antimicrobial therapy. Both, delayed antibiotic administration[2] and use of inappropriate antimicrobial therapy[3-5] is associated with an increased mortality in patients, Furthermore, inappropriate use of antibiotics has led to emerging antibiotic resistance amongst microbial pathogens, which poses as a global health concern causing prolonged illness, greater risk of death and increased risk of infection spread[6, 7]. The detection limit of the most optimal DNA extraction procedure in our studies was 50 colony-forming units (cfu) per ml of whole blood. We found that the removal of inhibitory human DNA, bacteria enrichment and use of a larger volume of blood improved the rate of successful pathogen recovery from whole blood. Though, the question remains whether this detection limit is sufficient for bacterial identification from direct specimens. Therefore, we conducted a pilot study to investigate the bacterial load in pediatric whole blood samples. Contradictory findings exist concerning the amount of circulating microorganisms in the bloodstream of pediatric patients. Although it was previously thought that the bacterial or fungal load was higher in children compared to adults[8] a report by Kellogg *et al.* presented the occurrence of low-level bacteremia in the majority of their population[9]. This can be problematic since the diagnostic yield is directly related to the volume of blood sampled in both adults[10-12] and children[13, 14]. For this purpose, 20 to 30 ml of blood per culture is recommended in adults[15, 16], but obviously cannot be obtained in children (<10 ml). Overall, a low amount of bacteria was found. In 85%, less than 50 cfu per ml of whole blood were present, which would be below the detection limit of our real-time PCR assay following an optimized DNA extraction protocol, and result in failure of detection.

In summary, we could show that the combination of different factors i.e. blood volume, human DNA elimination and bacteria enrichment can improve the detection limit of bacterial DNA extraction methods. On the other hand, our findings together with other reports demonstrated that the bacterial load in adult[8] and pediatric samples[9] are rather low, hampering the current capabilities of methods for bacterial detection directly from whole blood.

The detection and identification of bacterial and fungal pathogens

Following bacterial and/or fungal DNA extraction, different molecular techniques can be used for the detection and identification of microorganisms. Nucleic acid amplification methods such as real-time PCR can generate results within hours, thereby anticipating on the need for rapid diagnosis of infection. In order to enable detection of the most frequently observed bacterial microorganisms found in our hospital, a real-time PCR assay was designed for the identification of both Gram-positive (*Staphylococcus* spp., *Staphylococcus aureus*, *Enterococcus* spp., *Streptococcus* spp. and *Streptococcus pneumonia*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas* spp. and *Pseudomonas aeruginosa*). In addition, the presence or absence of any bacterial DNA (others than the targets in the panel) could be confirmed using a broad-range probe for the eubacterial 16S rDNA gene. Testing of 248 blood cultures resulted in an overall agreement of 97% with conventional culture-based bacterial identification **(Chapter 3a)**. Since the genotypic identification of microorganisms from whole blood can be hampered by the occurrence of low bacterial loads, giving rise to false-negative results, blood cultures were used as starting material for real-time PCR analysis. The identification of the included Gram-positive and Gram-negative bacterial targets could be established within two hours, which could be used as indicator for the guidance of antimicrobial therapy. A similar approach was used for the design of a real-time PCR assay identifying four clinically relevant *Candida* spp. from blood culture material **(Chapter 3b)**. Invasive infections with *Candida* spp. have been associated with high mortality rates, ranging from 38% to 74%[17]. In a pilot experiment, blood cultures which presented with fungal cells in Gram-staining, were tested with the molecular assay. All *Candida*-positive blood cultures were correctly identified at species level. In addition, the assay was able to identify *Candida albicans* in blood culture bottles which remained culture-negative, emphasizing the additive effect of molecular identification to phenotypic identification.

In both real-time PCR assays, priority was given to the genera or species most frequently found in blood cultures and/or those that could direct the choice of a suitable antibiotic therapy. For instance, in fungal infections, the detection of non-albicans *Candida* spp. is important because of the observed reduced susceptibility to frequently used antifungal agents. The use of a limited target panel did not offer a full laboratory report defining the exact etiologic agent, but did offer preliminary results covering bacterial pathogens which are frequently observed and this within two to three hours from positively flagged blood cultures. In contrast, conventional culture-based identification involving Gram-staining and biochemical testing, requires the generation of single colonies, usually reached after overnight growth. In the past, many broad-range PCR

assays were combined with time-consuming sequencing of the 16S rDNA region[18-21]. More recently, commercial assays such as Septi*Fast* (Roche Diagnostics, Mannheim, Germany) and SepsiTest (MolZym, Bremen, Germany) were developed and evaluated[21-26], and are intended for analysis of whole blood samples. These methods enable the detection of 25 to 40 sepsis-related pathogens, and can be completed within six to eight hours. An alternative is the Prove-it Sepsis (Mobidiag, Helsinki, Finland), a microarray-based system detecting 80 pathogens from blood cultures, and this within 3.5 hours[27]. Although these diagnostic tools offer a more extended panel of targets, and some can be directly applied to whole blood samples, more studies will have to be performed to assess their implementation in routine diagnostics. Until now, these can only be used in parallel to conventional culture-based identification, and thus lead to more costs. Our bacterial and fungal multiprobe assays were adapted to the prevalence of the most frequently encountered bacteria and fungi in our hospital, but can be streamlined and adjusted, offering both flexibility and reduced costs. Taking into account a 15 hours median time to positivity of blood cultures[28-30], the turnaround time until initial results using a real-time PCR assay was approximately 18 hours. In the past, traditional phenotypic manual identification methods required a turnaround time until final result of approximately 63 and 72 hours for bacteria and fungi, respectively[31]. Nowadays, automated microdilution broth assays (e.g., Vitek (BioMérieux), Phoenix (Becton Dickinson) are used, offering full pathogen identification and antibiotic susceptibility testing (AST) with a turnaround time of 41.5 - 48 hours[31]. Next, we suggested that the determination of the bacterial load could also be of clinical value for other sample materials or applications **(Chapter 4)**. For instance, quantification of uropathogens is essential as different bacterial loads ($>10^3$ or 10^5 cfu/ml) are used in combination with clinical symptoms to identify urinary tract infections (UTIs) in different population groups[32]. The multiprobe real-time PCR assay presented in Chapter 3 was now used for both identification and semi-quantification of uropathogens. Our findings have shown the capability to categorize into no bacteriuria ($<10^3$ cfu/ml), low-level bacteriuria (10^3-10^4 cfu/ml) and significant bacteriuria ($\geq 10^5$ cfu/ml). Additionally, the molecular screening of urinary specimens would have reduced the amount of urine specimens to be cultured with 47%. The potential of new less-time consuming methods was shown in previous studies[33-36], especially since up to 80% of urine cultures are negative[37].

To conclude, the development of rapid and highly specific real-time PCR assays has enabled identification and semi-quantification of microorganisms starting from positive blood cultures and urine specimens within two hours. Both assays were designed for clinically important bacteria and fungi and showed to be promising tools for the rapid and accurate diagnosis of infections, Implementation of these novel assays in the clinical microbiology laboratory

could be beneficial for the patients' outcome in terms of rapid and appropriate therapy, but clinical studies are obligatory to assess the value (e.g., reduction in time to diagnosis, modification of therapy, hospital lengths of stay) of rapid diagnostics in patients settings.

The genotypic detection of staphylococcal antibiotic resistance

During recent years, a wide range of diagnostic options for the rapid identification of bacterial and fungal microorganisms from different clinical samples were developed. However, the role of conventional culture-based methods remained prominent, not only because of the inability of each novel test to be implemented as unique tool for identification and solely replace culturing, but also because of the need for further subculturing to perform antimicrobial susceptibility testing (AST). Because of the large amount of genes involved in antibiotic resistance, microarray-based technologies, in which gene-specific probes are deposited on a solid surface, were presented as promising tools for the detection of large panels of antibiotic resistance genes[38, 39]. However, these are often not appropriate in terms of costs,laboratory equipment and user-friendly format[40]. As a proof of principle, we developed a real-time PCR offering a panel of the most requested and clinically relevant antibiotics to treat staphylococci and found it to be highly correlated with phenotypic AST methods **(Chapter 5)**. The assay fulfilled the selection criteria for an acceptable AST system (>90% categorical agreement) stated by Jorgensen[41], and could be performed within two hours starting from a positive blood culture. In our study, the use of blood cultures was of added value since previous reports that focused on antibiotic resistance detection in staphylococci were mainly performed on bacterial suspensions after subculturing[42-46].

To conclude, if genotypic antibiotic resistance in staphylococci was combined with genotypic identification, as presented in chapter 3a, the turnaround time to results could be drastically reduced. Gaining knowledge about the etiologic agent together with a partial antibiotic resistance profile could enable more adequate therapy guidance at an earlier time point compared to conventional identification and AST. However, this field of diagnostics is still in its infancy, requiring further investigation in terms of antibiotic resistance mechanisms and prevalence of antibiotic resistance determinants. Currently, the genotypic approach can provide more rapid detection of well-established antibiotic resistance determinants such as *mecA*[47], particularly of importance in the control and surveillance of methicillin-resistant *S. aureus* (MRSA), but cannot solely replace the phenotypic methods used in routine AST.

Conclusions and future perspectives

In summary, the data presented in this thesis show the potential of molecular testing methods for the detection and identification of bacterial and fungal microorganisms in terms of rapidity, sensitivity and specificity. Also, in order to achieve maximum pathogen recovery from whole blood samples, the importance of an optimal DNA extraction method was demonstrated. Although we established an improvement in bacterial detection limit by the elimination of human DNA, and the use of larger sample volumes, further studies comparing the positivity rate between blood cultures and whole blood samples should be done in order to assess the use of direct patient specimens (i.e., without enrichment by culture). As addressed in previous papers, the administration of adequate antimicrobial therapy is correlated with a decrease in mortality[4, 48-50]. From that perspective, the developed real-time PCR assays for the detection and identification of bacteria and fungi, together with the detection of important antibiotic resistance determinants could be important for the rapid diagnosis of certain infectious diseases. Overall, they achieved a drastic reduction in turnaround time, and offered a high sensitivity and specificity that could contribute to an improvement of current pathogen diagnostics in the clinical microbiology laboratory. Though, future studies are required that support and elaborate our findings, and prove their usefulness in clinical settings.

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Samenvatting

Een grote verscheidenheid aan bacteriën, virussen, schimmels en parasieten is verantwoordelijk voor de naar schatting 15 miljoen sterfgevallen per jaar als gevolg van infectieziekten. Infectieziekten gaan gepaard met een hoge morbiditeit en mortaliteit en vormen bijgevolg een hoge sociaal-economische last voor de maatschappij. De bestrijding van infectieziekten is complex en afhankelijk van verschillende aspecten, waaronder preventie, diagnose en behandeling.

Het slagingspercentage van de behandeling wordt beïnvloed door diverse factoren, waaronder de snelheid en de precisie van de diagnose en daarom zijn snelle en gevoelige methoden voor de diagnose van infectieziekten essentieel. Nieuwe diagnostische technieken moeten bijdragen tot het voorkomen van sterfgevallen en tot het verbeteren van de kwaliteit van leven. Bovendien is een snelle interventie cruciaal om verspreiding van de ziekte te voorkomen en om de ontwikkeling van resistente stammen van bepaalde ziekteverwekkers te vermijden. Hoewel de op kweek gebaseerde methoden algemeen beschouwd worden als de gouden standaard voor de detectie en identificatie van ziekteverwekkers, dragen deze ook nadelen zoals het (relatief) lange tijdsbestek tussen de afname van een patiëntstaal en het verkrijgen van de uitslag van het microbiologisch onderzoek van moeilijk groeiende micro-organismen met zich mee. Moleculaire methoden bieden een alternatieve strategie, waarbij er zowel op niveau van eiwitten als nucleïnezuren wordt gezocht naar onderscheid tussen de verschillende soorten micro-organismen. Studies hebben aangetoond dat deze alternatieve methoden een verhoogde sensitiviteit en specificiteit bezitten, en daarnaast kunnen ze ook een aanzienlijke winst in tijd opleveren. Toch zijn er verdere studies en ontwikkelingen nodig om onder meer het gebrek aan onderscheid tussen dode en levende micro-organismen, en het gebrek aan een snelle bepaling van het antimicrobiële resistentie profiel te elimineren.

De studies uitgevoerd en beschreven in deze thesis zijn gericht op het ontwikkelen van moleculaire methoden voor de snelle diagnose van potentieel pathogene bacteriën en schimmels. Verschillende technieken, gaande van extractie tot detectie en identificatie van het bacteriële of fungale DNA, werden ontwikkeld, geoptimaliseerd en gevalideerd op verschillende klinische patiëntstalen.

De extractie van bacterieel en fungaal DNA uit verschillende lichaamsvloeistoffen

In de eerste studies werd er ingegaan op het belang van preanalytische methoden voor de selectieve extractie van microbieel DNA uit volbloed **(hoofdstuk 2a, 2b).** De implementatie van een snelle, moleculaire test biedt

immers een aanzienlijke winst in tijd wanneer deze rechtstreeks toepasbaar is op volbloed. De detectiegrens van de meest optimale DNA extractie methode bedroeg 50 kolonievormende eenheden (kve) per ml volbloed. Zowel het elimineren van remmende substanties aanwezig in bloed, als het gebruiken van grotere volumes zorgden voor een toename van de sensitiviteit. Om na te gaan of deze detectiegrens geschikt is voor klinische stalen, werd de bacteriële concentratie onderzocht in pediatrische volbloedstalen. Voorheen werd er gesuggereerd dat de concentratie micro-organismen in het bloed hoger was bij kinderen dan bij volwassenen. Maar er bestaan echter ook tegenstrijdige bevindingen die wijzen op het voorkomen van zeer lage aantallen van micro-organismen bij kinderen. Dit kan problematisch zijn omdat de diagnostische opbrengst direct gerelateerd is aan de hoeveelheid bloed die afgenomen wordt. Bij volwassenen is het aanbevolen om 20 tot 30 ml bloed per bloedkweekfles af te nemen, maar bij kinderen bedraagt dit minder dan 10ml. Tijdens ons onderzoek werden er lage aantallen bacteriën gevonden in bloedstalen van kinderen. In 85% van de stalen waren er minder dan 50 kve per ml bloed aanwezig, wat erop wijst dat onze geoptimaliseerde DNA extractie methode in deze gevallen geen ziekteverwekker zou aantonen. In conclusie, de combinatie van verschillende factoren d.w.z. groter bloed volume, het elimineren van remmende substanties (m.n. humaan DNA) en de verrijking van bacterieel DNA heeft geresulteerd in het verbeteren van de detectiegrens van bestaande DNA extractie methoden die worden aangewend voor de diagnose van ziekteverwekkers. Daarnaast hebben we aangetoond dat de bacteriële concentratie in kinderen net zoals bij volwassenen eveneens laag kan zijn, wat het gebruik van deze methoden voor de rechtstreekse detectie van micro-organismen rechtstreeks uit volbloed belemmert.

De detectie en identificatie van bacteriële en fungale micro-organismen

Nadat het bacteriële of fungale DNA wordt geïsoleerd uit het patiëntstaal, kan het opgezuiverde materiaal gebruikt worden voor de volgende stap in de analyse, namelijk de precieze identificatie van de ziekteverwekker. Hiervoor worden moleculaire technieken zoals de polymerase ketting reactie (PCR) toegepast. Deze is gebaseerd op het vermenigvuldigen van het aanwezige DNA tot een analyseerbare concentratie. Deze methode is uitermate snel, en kan binnen enkele uren resultaten genereren, waardoor een snelle detectie van pathogenen mogelijk is. In een van onze studies werd er een PCR methode ontwikkeld om de meest voorkomende bacteriële ziekteverwekkers op te sporen in bloedkweken **(Hoofdstuk 3a)**. De test was bestemd om in de eerste plaats een bacteriële infectie te diagnosticeren. Daarnaast is het mogelijk om met deze test de volgende micro-organismen te identificeren: *Staphylococcus*

species, *Staphylococcus aureus*, *Enterococcus* species, *Streptococcus* species, *Streptococcus pneumonia*, *Escherichia coli*, *Pseudomonas* species en *Pseudomonas aeruginosa*. Er werden 248 bloedkweken onderzocht op aanwezigheid van bacteriën, en in 97% konden de resultaten van de referentiemethode (kweekgebaseerde identificatie) bevestigd worden met onze nieuwe identificatietest. De analyse met behulp van deze nieuwe methode neemt in totaal 2 uur in beslag, hetgeen een drastische verbetering betekent ten opzichte van de huidige bacteriële diagnostiek die 18 - 24 uur in beslag neemt.

Een soortgelijke test werd er ontwikkeld voor de diagnose van relevante schimmelinfecties, veroorzaakt door Candida species **(Hoofdstuk 3b)**. Invasieve infecties met Candida species zijn geassocieerd met hoge sterftecijfers, variërend van 38% tot 74%. In onze experimenten werden alle Candida-positieve bloedkweken correct geïdentificeerd op species niveau. Daarbij zijn we er met onze nieuwe methode in geslaagd om *Candida albicans* te identificeren in bloedkweken die als negatief werden beschouwd met de referentiemethode. Het is alom bekend dat schimmels vaak traag en moeilijk te kweken micro-organismen zijn, waardoor onze alternatieve, moleculaire methode een belangrijk voordeel oplevert ten opzichte van de kweekgebaseerde methode. Ook moleculaire methoden hebben in de loop der jaren een grondige evolutie doorstaan. In het verleden werd de PCR techniek voornamelijk gecombineerd met een tijdrovende sequentieanalyse van het bacteriële 16S rDNA gebied. Hier kwam recent verandering in door het op de markt brengen van commerciële kits zoals Septi*Fast* (Roche Diagnostics) en SepsiTest (Molzym) voor de analyse van bloedstalen. Deze methoden zijn bestemd voor de detectie van 25 tot 40 sepsis-gerelateerde pathogenen, en kunnen worden afgerond binnen de 6 tot 8 uur. Prove-It Sepsis (Mobidiag) is een gelijkaardig systeem voor de diagnose van 80 micro-organismen uit bloedkweken, en dit binnen de 3,5 uur. Hoewel deze veelbelovende technologieën een meer uitgebreid panel van micro-organismen aanbieden, en sommigen zelfs rechtstreeks kunnen worden toegepast op volbloedstalen, zullen studies hun mate van implementatie in routine diagnostiek nog moeten uitwijzen. Ondanks alle onderzoek is het duidelijk dat de huidige moleculaire technieken nog niet voldoende in staat zijn om de kweekgebaseerde referentiemethoden te vervangen.

Naast identificatie van de ziekteverwekker is het in bepaalde gevallen ook noodzakelijk om kennis te hebben over de mate van infectie, met andere woorden de hoeveelheid micro-organismen aanwezig in het patiëntstaal. In een studie waarbij we gekeken hebben naar urineweginfecties, hebben we getracht om zowel identificatie als kwantificatie van het micro-organisme te bewerkstellingen **(Hoofdstuk 4)**. De bepaling van de hoeveelheid micro-organismen is essentieel omdat verschillende afkappunten ($>10^3$ of 10^5 kve/ml) worden gebruikt in combinatie met klinische symptomen als diagnose van

urineweginfecties in verschillende bevolkingsgroepen. Het toepassen van de nieuwe moleculaire methode gebruik makende van de PCR laat ons toe om te categoriseren in: normale urine (< 10^3 kve/ml), bacteriurie (10^3-10^4 kve/ml) en significante bacteriurie (≥10^5 kve/ml). Bovendien zou deze snellere screening van urinestalen ervoor zorgen dat identificatie met behulp van kweek gereduceerd wordt met 47%.
In conclusie, de ontwikkeling van een snelle, sensitieve en specifieke real-time PCR methode heeft de identificatie en semi-kwantificatie van klinisch relevante bacteriën en schimmels uit bloedkweken en urine mogelijk gemaakt, en dit binnen de 2 uur. Er is echter nog wel behoefte aan klinische studies die de waarde (impact van m.n. reductie in tijd tot diagnose en versnelde bijstelling van therapie, hospitalisatieduur, complicaties,…) en rol van moleculaire methoden in het verbeteren en versnellen van zowel diagnose als behandeling van patiënten aantonen.

De genotypische detectie van antibiotica resistentie in *Staphylococcus* species

Veel studies in het verleden hebben zich toegelegd op het ontwikkelen van een snelle methode voor de identificatie van bacteriële en fungale infecties uit verschillende patiëntstalen. Toch is steeds de rol van de kweekgebaseerde referentiemethode prominent aanwezig gebleven, ten gevolge van het gebrek aan een alternatieve methode voor het bepalen van een antibiogram. Het ontwikkelen van een alomvattende genotypische test voor antibioticaresistentie wordt bemoeilijkt door het voorkomen van de grote hoeveelheid genen die betrokken zijn in de verschillende resistentiemechanismen. Eerdere studies gebruik makende van micro-arrays hebben laten zien dat deze technologie geschikt is voor dergelijke toepassingen. Het feit ook dat deze technologie een hoge capaciteit heeft, maakt hem aantrekkelijk voor dit type onderzoek. Vaak is deze micro-array technologie echter niet geschikt in termen van kosten, benodigde laboratoriumapparatuur en gebruiksvriendelijkheid.
In het laatste experimentele hoofdstuk hebben we getracht een PCR methode te ontwikkelen voor de bepaling van antibioticaresistentie in staphylococcen **(Hoofdstuk 5)**. Een panel van de meest gevraagde en klinisch relevante antibiotica werden zowel genotypisch als fenotypisch bepaald. De resultaten voldeden aan de selectiecriteria (> 90% categorische overeenkomst tussen referentiemethode en nieuwe test) opgesteld door Jorgensen, en waren beschikbaar binnen de 2 uur nadat een bloedkweek positief bevonden was. Het aanbieden van zowel identificatie van het micro-organisme als bepaling van resistentie kan de analyse sterk versnellen en stelt de aanvrager in staat de keuze van de juiste therapie te starten op een eerder tijdstip dan verkregen na de conventionele kweekgebaseerde methode. We moeten ons echter bewust

zijn dat het onderzoek naar moleculaire methoden voor antibiotica resistentie bepaling zich in een beginstadium bevindt en dat nog meer studies vereist zijn wat betreft resistentiemechanismen en antibiotica resistentie determinanten. Toch bestaan er reeds enkele voorbeelden van genotypische testen die reeds in de kliniek toegepast worden zoals de detectie van het mecA gen, karakteristiek voor methicilline-resistente *Staphylococcus aureus* (MRSA). De verwachting is dan ook dat dit in de toekomst voor meerdere resistente micro-organismen het geval zal zijn.

Conclusie

Samenvattend hebben onze bevindingen aangetoond dat moleculaire methoden voor wat betreft snelheid, sensitiviteit en specificiteit de huidige referentiemethoden kunnen overtreffen en zeker kunnen aangewend worden voor de detectie en identificatie van bacteriële en fungale micro-organismen. We hebben kunnen aantonen dat bepaalde elementen in het DNA extractie proces, met name grotere bloedvolumes, en het elimineren van humaan DNA, de detectiegrens van moleculaire testen drastisch kan verlagen. Daarnaast heeft de ontwikkeling van een identificatietest voor zowel klinisch relevante bacteriën als schimmels een snelle analyse (binnen één werkdag) van patiëntstalen zoals bloedkweken mogelijk gemaakt. Al deze verbeteringen moeten er op termijn toe leiden dat zowel de diagnose als de behandeling van patiënten met verdenking op infectie sneller verlopen, waardoor ook de morbiditeit en mortaliteit zal afnemen. Er zullen echter meer studies moeten uitgevoerd worden waarbij de implementatie van deze testen onderzocht wordt, en of het op termijn mogelijk zal zijn om patiëntstalen rechtstreeks te analyseren, zonder voorafgaande kweek.

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